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B. C. J. G. KNIGHT A. A. MILES

Associate Editors

G. C. AINSWORTH
A. T. R. MATTICK
W. B. BRIERLEY
K. M. SMITH
T. GIBSON
A. W. STABLEFORTH
D. D. WOODS

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Observations on the Differential Inhibition of Coliform Bacilli and Rough Variants of Intestinal Pathogens

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SUMMARY: Differential inhibition of Gram-negative bacilli of intestinal origin appears to depend upon a combined action of bile salts and electrolytes. This occurs only under certain conditions, among which aerobic incubation is one and the nature of the surface upon which the cultures are grown is another.

During the recent war desoxycholate could not be obtained in Great Britain and a modified Leifson's agar medium (1935) was prepared, substituting 'taurocholate' for desoxycholate, rosolic acid for neutral red and a peptic digest of sheep serum for the pork infusion peptone nutrient base. This medium gave adequate inhibition of *Bacterium coli* (*Escherichia coli*), while allowing good growth of smooth variants of intestinal pathogens. Rough variants of these micro-organisms were markedly inhibited.

Materials and methods

In all experiments the reaction of the media was standardized at pH 7·6. Plates were dried for 30 min. at 37° before use. The six strains of micro-organisms tested were inoculated thus: A 3 mm. loopful of a 24 hr. agar culture was suspended in 9 ml. of distilled water and well mixed; this suspension was stroked on the plates by dipping into it a T-shaped piece of 0·018 in. gauge chrome iron wire, the width of the spreading surface being 3 mm. This inoculation of a small quantity of a well-diluted culture was adopted to ensure that only a minimal amount of nutrients would be carried over from the parent growth.

The taurocholate used was the sodium tauro-glycocholate marketed by British Drug Houses Ltd., of B.P.C. standard. All quantities quoted as % subsequently refer to the weight of the substance in 100 ml. of solution, i.e. % (w/v).

The following strains of organism were used for testing the variously compounded media:

SR, a rough variant of a recently isolated Sonne III bacillus.
SS, a smooth variant of the same micro-organism.
C1, *Bacterium coli communis* from human faeces.
C2, *Bact. coli communior* from human faeces.
A1, *Aerobacter* sp. from milk.
A2, *Aerobacter* sp. from water.

The standards adopted for comparing the inhibitions of growth were: (*a*) 'partial differentiation' which means that strains SR, SS, A1 and A2 grew, but C1 and C2 failed; (*b*) 'complete differentiation' means that SS grew, but all others failed to do so.

Comparison of taurocholate and desoxycholate media

The first experiment was designed to compare desoxycholate and taurocholate in presence of citrate. Four batches of modified Leifson's agar medium (Brodie, 1942) were prepared, all containing bile salts (0·5 %) and trisodium citrate (2·5 %), but without ferric ammonium citrate and rosolic acid (as it was desired to test only the essential constituents of the medium), as follows: A, serum peptic digest + taurocholate + citrate; B, serum peptic digest + desoxycholate + citrate; C, meat infusion peptone + taurocholate + citrate; D, meat infusion peptone + desoxycholate + citrate.

The results of one experiment were that all strains grew on medium C, that media A and D inhibited all but strain SS, and that medium B inhibited all strains. From this it would appear that taurocholate is less effective than desoxycholate as a differentiating agent, but, when the basal nutrient is peptic digest of serum, taurocholate can replace desoxycholate. Other results were not always so clear-cut, though similar ones were obtained on very many occasions. Occasionally the degrees of inhibition were less marked, media A and C giving partial differentiation, medium B complete differentiation and medium D growth of SR and SS only. From this it appears that changes of the basal nutrient might alter the effect of the bile salts. The difference between the media is therefore probably quantitative, not qualitative.

Effect of electrolytes

Leifson apparently used citrate to give the desired inhibition of *Bact. coli*, but he noted that sodium acetate and even sodium chloride produced a similar effect. He suggested that citrate was suitable because it forms a salt with desoxycholate. Media containing peptic digest of serum have a sodium chloride content of about 0·9 % (Brodie, 1942), which is rather more than is present in most media, and it is probably upon this high salt content that the satisfactory results obtained with the modified Leifson's medium depends.

Sodium citrate in absence of bile salts. When sodium citrate was used in the absence of bile salts inhibition occurred with increasing citrate content but was not distributed among the strains tested in the same order as that produced by bile salts + citrate. All six strains grew in the presence of 4 % citrate; only SR and C2 failed to grow on exposure to 5 % citrate. It seems, therefore, that the bile salts in the medium do not merely enhance the action of electrolytes.

Sodium citrate, sodium aconitate and sodium fumarate. Varying concentrations of these sodium salts, representing two trivalent and one divalent organic anions, were used with a medium in which the peptic digest of serum was diluted to one-quarter the strength employed in routine work. The diluted digest was used because preliminary tests of quarter-strength digest as the basal nutrient in presence of 0·5 % taurocholate and 0·5 % NaCl yielded clear-cut partial differentiation with 2·5 % citrate and complete differentiation with 5 % citrate.

The same degree and distribution of partial differentiation was produced by 0·1 M-citrate, 0·1 M-aconitate and 0·2 M-fumarate; complete differentiation was given by 0·2 M-citrate, 0·2 M-aconitate and 0·3 M-fumarate. As shown by Leifson, the effect of citrate is therefore not specific.

Investigation of salts in relation to their place in the Hofmeister series. The previous observations suggested that the influence of electrolytes might be largely physical and related to their position in the Hofmeister series. 'Swelling' or 'shrinking' of the agar upon which the organisms were grown, or a kindred effect upon the bacilli themselves, might be a factor determining the differential inhibition.

Table 1. *Molar concentrations of salts giving differentiation when added to* 2 % *agar medium containing quarter-strength peptic digest of serum and* NaCl (0·5 %) *in presence of* (*a*) *taurocholate* 0·5 %, *and* (*b*) *desoxycholate* 0·5 %. *Plates incubated aerobically at* 37·5°

		Concentration of salts needed for	
		Partial differentiation (M)	Complete differentiation (M)
(*a*)	Ammonium citrate	0·057	0·1
	Potassium citrate	0·066	0·12
	Sodium citrate	0·1	0·2
	Ammonium sulphate	0·13	>0·3
	Potassium sulphate	>0·2	—
	Sodium sulphate	0·3	0·42
	Ammonium chloride	0·43	0·48
	Potassium chloride	0·5	0·57
	Sodium chloride	0·53	0·66
	Ammonium acetate	0·125	0·166
	Sodium acetate	0·33	0·5
(*b*)	Ammonium citrate	0·04	0·06
	Sodium citrate	0·08	0·12
	Sodium sulphate	0·18	0·33

Various salts (Table 1) were added in varying concentrations to the quarter-strength peptic digest agar containing taurocholate 0·5 % and NaCl 0·5 % (which is the NaCl content of Saunders, Dorfman & Koser's (1941) synthetic medium). Table 1 (*a*) records the molar concentrations of electrolyte required for partial and complete differentiation. Similar tests were made with desoxycholate 0·5 % in place of taurocholate (Table 1 (*b*)); the results indicate that desoxycholate differs quantitatively from taurocholate in its action.

A further test was made of meat infusion peptone agar of unknown electrolyte content as nutrient. Both taurocholate and desoxycholate were used. The results were substantially the same as those with the peptic digest of serum except that slightly higher molar concentrations were required. Since with both nutrient media desoxycholate was more active than taurocholate it is possible that both the bile salts acted in the same way. It is possible that the activity of the serum peptic digest as compared with the meat infusion peptone medium was due to its higher NaCl content. Complete differentiation was obtained in presence of 0·75 M-NaCl in quarter-strength peptic digest containing 0·5 % taurocholate.

These observations suggest that there is some correlation between the position of the various salts in the Hofmeister series and their power to inhibit coliform bacilli and rough variants of the intestinal pathogens in presence of bile salts.

Effect of the nutrient medium

The two basal media so far employed were substantially the products of peptic digestion. The effect of taurocholate and citrate added to other types of nutrient was tested as follows. Nutrient agars were prepared with (1) Douglas broth; (2) tryptic digest of serum; (3) tryptic digest of casein as used for the preparation of Gladstone & Fildes (1940) 'CCY' medium; (4) acid hydrolysate of casein as prepared for the same medium; (5) yeast extract as used in 'CCY' medium; (6) the complete 'CCY' medium.

Complete differentiation was obtained in the presence of 0·5 % taurocholate and 5 % citrate with all the nutrient basal media tested except the complete 'CCY' medium which, containing sodium glycerophosphate and sodium lactate, required only 3·75 % of citrate. Provided a nutrient medium is adequate to support the growth of the strains tested, the differential effect can be obtained by adjusting the electrolyte content of the medium in the presence of bile salts. When the salt mixture of Saunders *et al.* (1941) was incorporated in agar containing 0·5 % taurocholate and 2·5 % citrate, and the NaCl content raised to 0·9 and 1·3 %, partial and complete differentiation respectively were obtained.

Fractionation of peptically digested serum

Ethanol fractionation. Various ethanol fractions of the peptically digested serum were tested in an attempt to define the distribution of any factor which might influence the differential effect. Material soluble in 70 % (v/v) ethanol-water was an excellent nutrient medium, as sharp in its differential action in the presence of 0·5 % taurocholate and 2·5 % citrate as the crude digest. The corresponding precipitate was a poor nutrient and devoid of differentiating activity. The fractions soluble in 80, 90 and 100 % (v/v) ethanol when used to compound media showed some differentiating action, but full activity was regained only when the corresponding filtrates and precipitates were again mixed.

Ash from peptic digest. The ash from a known quantity of peptically digested serum was reconstituted to the original volume. When quarter-strength peptic digest of serum was replaced in agar media by its content of ash in the presence of 0·5 % taurocholate and 2·5 % citrate, partial differentiation was obtained of the same degree as was achieved by using quarter-strength digest, 0·5 % taurocholate and 2·5 % citrate. The indication was that the differentiating power of the medium was dependent upon a suitable balance of nutrients, bile salts and electrolytes.

Surface-active agents other than bile salts

Because of their surface activity the influence of the bile salts might be due to physical rather than to physiological effects. Other surface-active agents were therefore investigated. Sodium oleate, sodium ricinoleate, sodium oleyl sulphate, Tergitol, sodium succinate and Aerosol OT (diethylsulphosuccinate), all having surface-active anions, and sapamine, having a surface-active cation, were compared with bile salts in the presence of peptic digest of serum of routine strength (NaCl 0·9 %). With 2·5 % citrate and the surface-active agents in dilutions of 1/300–1/100, not even partial differentiation was obtained. It was noted, however, that: (*a*) strain SR was more susceptible than SS to ricinoleate, Tergitol, Aerosol OT and sapamine; (*b*) strains C1 and C2 showed some effect with Tergitol and Aerosol OT; (*c*) strains A1 and A2 were uninfluenced by any of the agents tested.

Similar experiments were conducted with 1/200 dilutions of the surface-active agents and different concentrations of citrate up to 5 %. Strain SR was inhibited by all the agents except sodium oleyl sulphate and sodium succinate above a citrate concentration of 2·5 %, but growth of the *Aerobacter* spp. remained maximal. It seems possible that the inhibition of SR as compared with SS may have been due to a combined effect of electrolyte and surface active agent. This does not hold for the other micro-organisms tested except that the commercial preparations Tergitol and Aerosol OT inhibited strains C1 and C2 when the concentration of citrate was increased. The action of bile salts differed from that of the other surface-active agents in that only bile salts inhibited the *Aerobacter* strains in presence of citrate.

The effect of air and the nature of the surface

In the experiments so far described the inoculations were made on the surface of 2 % agar media. The inhibition might be affected by the exposure to air or to a surface condensation of some of the constituents of the medium.

Anaerobic cultivation. Tubes of routine bile salt citrate medium (Brodie, 1942) were prepared and, when the surfaces were dry, the six cultures were investigated by stab inoculation. After 24 hr. at 37·5° the zone of visible growth extended up to and over the surface only with strain SS. With strain A2 it just reached the surface but not with the others. After 4 days' incubation A2 began to grow on the surface, and by this time strains C1, C2 and A1 had reached the surface. On the 5th day strain SR reached the surface and grew thereon, strains C1, C2 and A1 still failing to give surface growth.

Plates were made using routine strength peptic digest of serum (NaCl, 0·9 %) and 2·5 % citrate with added taurocholate (0·5 %) or desoxycholate (0·5 %). A similar set was made with meat extract peptone nutrient medium. After inoculation with the six strains of micro-organisms used, duplicate sets of plates were incubated aerobically and anaerobically. Only under aerobic conditions of incubation was the differential inhibition obtained.

Effect of varying the gel. All culture media so far employed contained agar as the gelling agent. A series of comparative tests was made with a number of different gelling agents. The quarter-strength peptic digest medium containing NaCl 0·5 %, taurocholate 0·5 % and citrate 2·5 % was solidified with: (*a*) agar 2 %, (*b*) substitute agar prepared from *Gigartina stellata* 2 %, (*c*) gelatin

15 %, (*d*) mixtures of agar and gelatin, and (*e*) silica gel. Aerobic incubation was used throughout.

With (*a*) and (*b*), at 22 and 37°, only SS grew well, SR, C1 and C2 were inhibited and growth of A1 and A2 was minimal. When gelatin was the solidifying agent the effect was reversed, SS growing least well of the six cultures tested. With gelatin, NaCl 0·5 % and citrate 2·5 %, in the absence of the peptic digest basal medium, the addition of taurocholate enhanced the nutrient quality of gelatin for the strains tested, whereas the inhibitory action of citrate was greater in the absence than in the presence of the nutrient base.

Table 2. *Degrees of differentiation obtained with agar, gelatin and mixtures thereof in the presence of serum peptic digest diluted* 1 *in* 4 *of that used in routine work* (NaCl *content adjusted to* 0·5 %), 0·5 % *taurocholate and* 2·5 % *sodium citrate. Plates were incubated aerobically at* 22°

Agar (2 %) parts	Gelatin (15 %) parts	Duration of incubation (days)	Strains tested					
			SR	SS	C1	C2	A1	A2
12	Nil	2	–	+	–	–	(+)	(+)
		8	–	+	–	–	+	+
10	2	2	5	+	–	–	+	+
		8	5	+	–	–	+	+
8	4	2	–	+	–	+	+	+
		8	9	+	±	+	+	+
6	6	2	3	+	+	+	+	+
		8	$\underline{\pm}$	+	+	+	+	+
4	8	2	–	+	+	+	+	+
		8	–	+	+	+	+	+
2	10	2	–	–	+	+	+	+
		8	–	–	+	+	+	+
Nil	12	2	–	–	+	+	+	+
		8	–	–	+	+	+	+

Figures indicate number of colonies.
– indicates no growth.
$\underline{\pm}$, ±, (+) to + indicate minimal to maximal growth.

Mixtures of agar and gelatin in presence of the other constituents yielded interesting results. These are shown in Table 2. Agar alone gave almost complete differentiation, gelatin allowed only the coliforms to grow; intermediate degrees of differentiation depended upon the amounts of each of the gelling agents present.

The findings when gelatin was used show that the influence of oxygen on differential inhibition was not manifested with all gelling agents.

Silica gel was prepared from commercial water-glass after the method of Anderson & Macsween (1942), but using hydrochloric in place of phosphoric acid for neutralization, and incorporated with quarter-strength peptic digest containing 0·5 % NaCl. Citrate (2·5 %) alone inhibited only strains SR and C2, a result comparable to that obtained with agar and the same salts. Taurocholate 0·5 % in the absence of citrate in silica gel inhibited all strains tested.

To obtain complete differentiation, it was found necessary to add only quarter-strength peptic digest of serum and 0·06 % taurocholate to the silica. It would appear that the high electrolyte content of commercial water-glass influences the differential inhibitory action of bile-salt media in which it is the solidifying agent. By adjusting the amount of bile salt, media prepared with it give the same differential inhibition of the micro-organisms tested as do agar media.

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Studies on Giant Amoeboid Organisms

1. The Distribution of *Leptomyxa reticulata* Goodey in Soils of Great Britain and the Effect of Bacterial Food on Growth and Cyst Formation

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SUMMARY: Giant amoeboid organisms may be isolated from soil and other materials by the use of suitable edible bacteria supplied on a base of non-nutrient agar.

Leptomyxa reticulata is widely distributed in the soils of Great Britain. The common occurrence of this organism in soils which have been unmanured or treated with artificial fertilizers for 100 years or more proves that it is a soil inhabitant.

Degrees of pH between 4·1 and 8·7 had no effect on the abundance of growth when a suitable bacterial strain was supplied as food on non-nutrient agar.

Ninety-two very varied strains of bacteria tested as food for *L. reticulata* varied greatly in edibility. Bacteria producing red, violet and blue pigments were mostly inedible. There was no correlation between Gram-staining and edibility.

Certain bacterial strains induced the formation of cysts by *L. reticulata*. This property was not correlated with their edibility.

The use of the recently improved dilution culture method of counting holozoic soil Protozoa (Singh, 1946*a*, *b*), based on the use of edible bacteria as food supply (Singh, 1941, 1942, 1945), revealed the occurrence of giant amoeboid organisms (*Leptomyxa reticulata* Goodey, 1914) in important numbers in arable soils. Goodey (1915), using 0·5–1·0 % agar containing nutrient broth, isolated three new organisms from a few soils receiving large quantities of farmyard manure. Two of these (*L. reticulata* and *L. flabellata*) are multinucleate forms, and the third (*Gephyramoeba delicatula*) is uninucleate. These interesting organisms, though little studied, have been classified by Goodey as proteomyxan rhizopods and placed in two new genera. Until the life cycles of these forms are completely worked out an attempt at classification is premature. An account on the nuclear division and cyst formation in *Leptomyxa reticulata* with certain remarks on its relationships will be published later.

Sandon (1927), in studies of the distribution of Protozoa fauna in soils of various parts of the world, could not find either *L. reticulata* or *L. flabellata*, although *Gephyramoeba delicatula* was recorded on a few occasions. During the past few years the writer has found *Leptomyxa reticulata* commonly present in arable soils. Sandon's failure to find *L. reticulata* and *L. flabellata* was probably due to the use of hay infusion and nutrient agar to isolate protozoa from soil. Goodey (1915) observed that in hanging-drop preparations in 1·0 % hay infusion and egg albumen, toxic substances produced by the bacterial activity check the growth of these organisms and finally kill them. It has been the experience of the writer during the past several years that agar containing nutrients is not satisfactory for the culture of protists which, however, develop normally on bacteria as the exclusive source of food supply (Singh, 1945, 1946*a*, 1947*a*, *b*). Nutrient media not only encourage the development of inedible micro-organisms but possibly also of those which produce toxic substances. Thus it is essential to use a non-nutrient substrate like plain agar or silica jelly and a suitable bacterial food supply in order to isolate and grow holozoic protists, and for quantitative studies, if reliable and consistent results are to be obtained. The present work deals with *L. reticulata* Goodey.

Method of isolation and culture

The method of isolation was similar to that used in the case of Acrasieae and myxobacteria (Singh, 1947*a*, *b*). One or two loopfuls of a readily edible bacterium growing on nutrient agar slope cultures (2–5 days old) are spread on the surface of non-nutrient agar (1·5 % washed agar in 0·5 % NaCl; pH 6·8–7·0) in the form of a disk or 'bacterial circle' of about an inch in diameter. It is preferable to use Gram-negative bacteria, because they do not encourage the development of lytic actinomycetes which are very commonly present in soils. Several such circles are made in each Petri dish. These circles are then inoculated at the centre either with small crumbs of soil, diluted soil suspensions or with small portions of some other substrate, and the plates are incubated at 21–23° for 2 weeks or more and examined at intervals under the low power of a microscope. In these crude cultures cysts of amoebae in large numbers, together with fruiting bodies of myxobacteria and *Dictyostelium* spp., are usually present in addition to the giant amoeboid organisms.

From the crude cultures giant amoeboid organisms are purified as follows. A portion of the agar containing these organisms, and very few or no amoebae and other micro-organisms mentioned above, are cut and transferred face downwards to freshly prepared 'bacterial circles'. Within 1 or 2 weeks large numbers of giant amoeboid organisms are seen in these cultures. By repeating this process a few times 'pure mixed' cultures of these organisms living on one species of bacteria are obtained. In order to obtain pure-line culture single cysts are picked with a micropipette from a suspension of cysts in 0·8 % NaCl, and each is inoculated to a 'bacterial circle' in a Petri dish. *Aerobacter* strain 1912 (Singh, 1941) was extensively used in the beginning of the work to isolate giant amoeboid organisms from various soils.

Distribution of Leptomyxa reticulata *in soil*

To study the occurrence of *L. reticulata* in soils, samples were taken from the top 2–6 in. The method of isolation and culture was that described above. Fifty-nine soil samples from Hertfordshire, Berkshire, Bedfordshire, Wiltshire, Kent, Cornwall, Glamorganshire, Breconshire, Pembrokeshire and Aberdeen were examined. *L. reticulata* was found in all the twenty-six arable soils. Of thirty-three grassland soils examined only twelve contained this organism. In addition to the soil samples mentioned above, nine soils of the classical plots of Barnfield and Broadbalk at Rothamsted were also examined. Some of these soils have been treated with farmyard manure, some with artificial fertilizers only and some have been unmanured, these treatments having been continued for about a hundred years. The presence of *L. reticulata* in all these plots proves that it is a true soil inhabitant. A few actively decomposing composts of straw and sludge that were examined also revealed the presence of *L. reticulata*. No correlation between the pH of the soil and the distribution of *L. reticulata* was found in soils ranging from pH 4·3 to 7·8. A few counts that have been made by the dilution culture method (Singh, 1946*a*) from Barnfield farmyard manured plot revealed the presence of *L. reticulata* up to the soil dilution of 1/1000.

Effect of pH *on the growth of* Leptomyxa reticulata

To test the effects of pH on the growth of *L. reticulata* 'bacterial circles' of *Aerobacter* sp. were made on 1·5 % non-nutrient agar adjusted to pH 4·2, 5·5, 6·0, 6·6, 7·0, 7·5, 8·3 and 8·7. *Leptomyxa reticulata* was inoculated in the centre of some of these bacterial cultures while the others were left uninoculated as controls. The plates were incubated at 20–21°. Within 7–10 days the bacterial cultures were completely consumed and large numbers of *L. reticulata* were present. At the end of 10 days bacteria from the control 'bacterial circles' were gently scraped off and a drop of the indicator was added to the agar in this area and another drop to the agar away from the centre. No change in the pH of the agar was produced by the presence of the bacteria. Similar tests showed that no change in the pH of the agar could be found where *L. reticulata* had grown with the bacterial associate for 10 days. Thus it is clear that pH values between 4·2 and 8·7 have no effect on the growth of *L. reticulata*.

Selection of bacterial food by Leptomyxa reticulata

Ninety-two strains of very varied bacteria were used. They comprise common and rare bacteria isolated from soil, plant pathogens (4752, 5945, 1989, 5944, 5942, 385, 5943, 387, 5241 and 1997; see Singh (1942) for the names of these strains), pigmented and non-pigmented bacteria mostly isolated from soil and a few strains of *Rhizobium*. The following pigmented species obtained from the National Collection of Type Cultures, Lister Institute, were also used (*N.C.T.C. Catalogue*, 4th ed. 1936): *Chromobacterium violaceum* (2537), *Sarcina lutea* (611), *Pseudomonas pyocyanea* (*Ps. aeruginosa*) (1999), *Micrococcus roseus* (2683) and *Sarcina aurantica* (952).

The strain of *Leptomyxa reticulata* was derived from a single cyst and was growing on *Aerobacter* sp. To test the selection of bacterial food by *Leptomyxa reticulata* the method of 'bacterial circle' described before was used. In each Petri dish two 'bacterial circles' were made on non-nutrient agar from a growth of the bacterium to be tested, derived from a 4–5-day agar slope culture usually on nutrient agar. Small pieces of agar containing *L. reticulata*, cut from actively growing cultures, were inoculated at the centres of these bacterial cultures, which were incubated at 19–20°. The plates were then examined under low power of a microscope after 7 and 15 days' incubation.

The bacteria tested fell into three groups; some of them were completely eaten (readily or slowly), others were partly eaten over a small area for a few days, after which *L. reticulata* either slowly died or formed multinucleate cysts; the remaining bacteria were either inedible or were eaten very slightly on rare occasions. *L. reticulata* completely consumed 44·5 % of the bacterial strains tested. Table 1 shows the relation between pigment formation by bacteria and the feeding reaction of *L. reticulata*. It is interesting to note that red, violet and green bacteria were not suitable food. This resembles the feeding reaction observed in the case of soil amoebae (Singh, 1945), except in the case of two red strains and a green one which were partly eaten by *L. reticulata*.

Table 1. *The relation of pigment production by bacteria to their edibility by* Leptomyxa reticulata

Bacterial strains	Total strains tested	Completely eaten (%)	Partly eaten (%)	Not eaten (%)
Colourless and yellow	65	52·3	24·6	23·1
Orange and brown	13	46·1	30·8	23·1
Red, violet, blue and green	10	0	30	70

Table 2. *The edibility of Gram-positive and Gram-negative bacteria by* Leptomyxa reticulata

	No. strains tested	Completely eaten	Partly eaten	Not eaten
Gram-positive	41	19	10	12
Gram-negative	52	23	15	14

In Table 2 is shown the relation between Gram-staining and the edibility of bacteria by *L. reticulata*. No correlation between edibility and Gram-staining exists, as was also the case with soil amoebae (Singh, 1945).

Table 3. *The difference in food specificity of* Leptomyxa reticulata *and a large soil amoeba tested on eighty-four varied strains of bacteria*

The edibility of the bacterial strains by the two organisms was similar for 62 % of the strains.

	% bacterial strains
Eaten by *L. reticulata* and by amoeba	29·7
Inedible to both	32·2
Eaten by *L. reticulata* but not by amoeba	13·1
Inedible to *L. reticulata* but eaten by amoeba	25

When the feeding reactions of certain holozoic organisms (two species of soil amoebae and myxoamoebae of two species of *Dictyostelium* and *Leptomyxa reticulata*) were compared on eighty-four strains of varied bacteria, it was found that *L. reticulata* differed from soil amoebae in 38 % cases and from myxoamoebae of *Dictyostelium* spp. in 47 % cases. The differences in its feeding reaction from a large soil amoeba are shown in Table 3.

Effect of bacterial food on the formation of cysts in Leptomyxa reticulata

Under suitable cultural conditions and with certain strains of bacterial food supplies a single large multinucleate individual will produce multinucleate cysts. Up to twenty or more cysts in clusters may be produced from each individual.

Table 4. *The production of multinucleate cysts by* Leptomyxa reticulata *on varied strains of bacteria*

Edibility of bacterial strains	No. bacterial strains tested	Relative amounts of cyst formation by *L. reticulata*: Large number	Few	None
Completely edible	40	15	8	17
Partly edible	25	5	6	14
Non-edible or slightly edible	27	0	9	18

The formation of cysts in the presence of pure cultures of bacteria was tested on non-nutrient agar as in the edibility tests. In Table 4 is shown the relation between edibility of bacteria and the formation of cysts. Among the forty strains of completely edible bacteria only fifteen lead to the production of cysts in large numbers. In the presence of the remaining twenty-five strains few or no cysts were formed. The same is true of the bacteria that were only partly consumed by *L. reticulata*. Inedible or slightly edible strains produced few or no cysts.

Aerobacter sp. (strain 1912) has been used for more than 3 years to keep the cultures of *Leptomyxa reticulata*. This strain is readily and completely consumed by *L. reticulata*, but no cysts are produced after a few subcultures. When the organisms had been grown for over a year on *Aerobacter* sp., subcultures being made every 2–3 weeks, they were supplied with twelve strains of bacteria, some completely and some partly edible, that normally induced the formation of numerous cysts. No cysts were produced on any one of these twelve strains supplied as food. During the past 2 years the original strain of *Leptomyxa reticulata* maintained on *Aerobacter* sp. has been tested twice to see if it could produce cysts on some of those bacteria on which it easily produced cysts when freshly isolated from soil, but without any success. Thus it seems that this strain of *L. reticulata* has lost its property of producing cysts after being subcultured on *Aerobacter* sp. for over a year. In the first few months of its isolation and culture on *Aerobacter* sp., it could produce cysts when grown in association with certain bacteria. A new strain recently isolated from soil on *Aerobacter* sp. easily produces cysts when fed with suitable bacteria. It may be of interest to mention in this connexion that the importance of certain bacteria for the production of cysts in the cultures of *Entamoeba histolytica* has been realized by several workers who have grown these amoebae on a diet of uncontrolled mixed bacterial cultures growing in a very rich medium. It has also been noticed frequently that under these conditions of growth *E. histolytica* loses its power to produce cysts. It is quite possible that these amoebae after growing for some time with bacteria unsuitable for cysts formation lose their property to produce cysts, as has been observed in *L. reticulata*.

DISCUSSION

The earlier work of Cutler, Crump & Sandon (1922) at Rothamsted showed that an inverse correlation existed between the numbers of bacteria and active amoebae in 85 % of the daily counts taken over a period of one year. Recently it has been shown (Singh, 1941, 1942, 1945) that soil amoebae do not feed indiscriminately on any bacteria. Besides true soil amoebae, other groups of holozoic organisms, such as species of *Dictyostelium* (Singh, 1947*b*, *c*) and giant amoeboid organisms, have been shown to be commonly present in arable soils of Great Britain. Since these groups of soil organisms feed selectively on bacteria like the true soil amoebae, it seems likely that they are of some importance, affecting not only the quantity but also the quality of the bacterial population of the soil. Although *Leptomyxa reticulata* has been found to be present only up to a soil dilution of 1/1000, its volume of more than 1000 times that of a soil amoeba suggests that it is important in soil economy.

This work was made possible by a grant from the Agricultural Research Council, to whom the author's thanks are due.

It is a great pleasure to express my sincere thanks to Dr H. G. Thornton, F.R.S., and Miss L. M. Crump for their interest in this work. Most of the strains of soil bacteria were given to me by Miss Crump from her personal collection.

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The Effect of pH at Different Temperatures on the Growth of *Bacterium coli* with a Constant Food Supply

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SUMMARY: The effect of pH over the range 5–9 on the growth of *Bacterium coli* with a constant food supply was studied at 20 and 30°. Total and viable counts were made and growth curves constructed. To discover the effects of starvation, sampling was continued after stopping the food supply.

The total count always substantially exceeded the viable. Each growth curve showed an 'initial phase' of varying daily increment in cell numbers merging into a 'steady phase' of roughly constant increment, which continued as long as food was supplied. Low pH slightly shortened the initial phase, low temperature greatly prolonged it. In the early initial phase development was slow at pH 5, but later became exceedingly rapid. Altogether, the conversion of the food supplied into (total) bacterial cells was best effected in conditions of low temperature and low pH, low temperature being the more important. These conditions also favoured high viable counts, and consequently smaller non-viability indices.

During starvation the apparent total counts declined, except at 30° and pH 5, when a steady increase occurred. Higher pH and lower temperature led to faster rates of decline. Viable counts remained approximately constant at pH 5, but otherwise the numbers declined.

Jordan & Jacobs (1944) showed that *Bacterium coli* could be cultivated successfully in an apparatus which permitted accurate control of temperature, pH and aeration, and enabled food to be added at a constant rate, so that the growth was controlled by the food supply. The apparatus was fully described and the results of preliminary experiments reported. After an initial phase, growth was shown to continue at an approximately steady rate so long as food was added. Subsequently, the apparatus was used to investigate the influence of temperature on the growth curves (Jordan & Jacobs, 1947), when it was shown that at pH 7 low temperatures were the most favourable to growth, in that they conduced to the formation of the largest amounts of bacterial cell substance from a given amount of food. Temperature also had a profound influence on the formation of viable cells. The proportion of viable cells formed was greatest at the lowest temperature used (15°), but at 35°, which is close to the temperature ordinarily regarded as the optimum for this organism, roughly half the cells formed were not viable, so that the number of viable cells remained virtually constant while the total cells steadily increased. At lower temperatures the viable count also increased. In view of these interesting findings it was decided to extend the experiments to determine how the cultures would behave under other conditions of pH. The results of this work are reported below.

METHODS AND RESULTS

Apparatus and technique. The apparatus and technique employed were fully described in the first paper of this series, and a brief summary was included in the second (Jordan & Jacobs, 1944, 1947). In the experiments described below the various pH values were all secured by the use of appropriate phosphate mixtures, since it was essential that different ions should not be introduced. Total counts were made by a turbidimetric method.

Experiments performed. Experiments were carried out at pH 5 and 6 at both 20 and 30°, and at pH 9 at 20°, daily estimations being made of the total and viable cells present. The rate of food addition was 0·066 ml./100 sec. of a solution of Difco dehydrated broth, equivalent to an actual addition of 15·2 mg. dehydrated broth/hr. After a time which varied between 14 and 21 days in different experiments the food supply was stopped, but sampling continued, in order that the effects of starvation could be studied. The results are too extensive for detailed presentation in tabular form, so they are shown graphically in Figs. 1–5.

General remarks. The main features of the growth curves at all pH values are similar and resemble those obtained when the effect of varying the temperature at a constant pH of 7 was being investigated (Jordan & Jacobs, 1947). They are, in brief, as follows: (1) The total count was always considerably higher than the viable. (2) An initial phase, in which the daily increments in the numbers of total and viable cells were changing rapidly, was succeeded by a steady phase, characterized by approximately constant increments. (3) There were indications that in the steady phase growth of the cultures had actually occurred in steps. (4) After the food supply was stopped, in most cases the numbers of viable cells and apparent total cells soon began to decrease.

Despite the tendency in the present experiments for the bacterial numbers to increase in steps during the steady phase, it was, as before, convenient to treat the data as if each culture had grown at a constant rate in that phase. The equations of the straight lines which best fit the data have been calculated (Table 2), and the lines drawn in Figs. 1–5 correspond to these. The linear treatment is justified by the smallness of the standard errors of these slopes (ΔT and ΔV respectively for the total and viable cells), but if the stepwise increase was real then the divergences from linearity were systematic and the standard errors have little significance. The slopes of the lines afford a useful assessment of the behaviour of the cultures as a whole, and enable a comparison to be made of the effects of the different conditions of pH and temperature. Also, as the sigmoid curve of growth during the initial phase should flow smoothly into the straight line of the steady phase, the latter has assisted in fixing an approximate duration for the initial phase. The end of that phase being coincident with the beginning of the steady phase, the equations representing the steady phase have been given in the appropriate form. The starvation phase has also been treated as if the cell numbers changed linearly with time, and in the figures smooth curves have been drawn to link the straight lines.

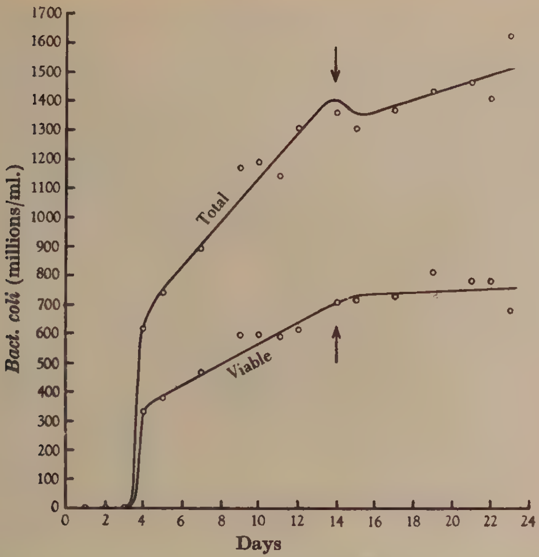

Fig. 1. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 30° and pH 5·0. The food supply was stopped shortly before taking sample marked by arrows.

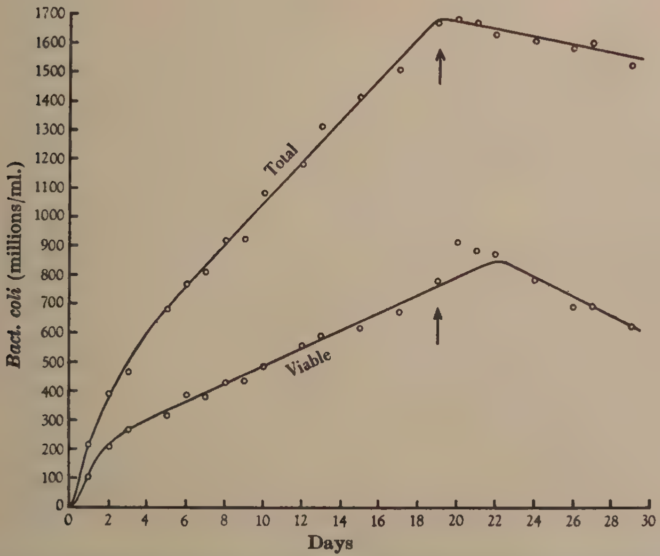

Fig. 2. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr at 30° and pH 6·0. The food supply was stopped shortly before taking sample marked by arrows.

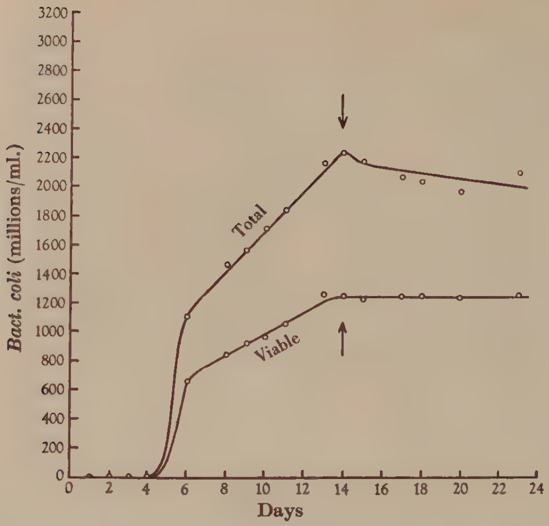

Fig. 3. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 20° and pH 5·0. The food supply was stopped shortly before taking sample marked by arrows.

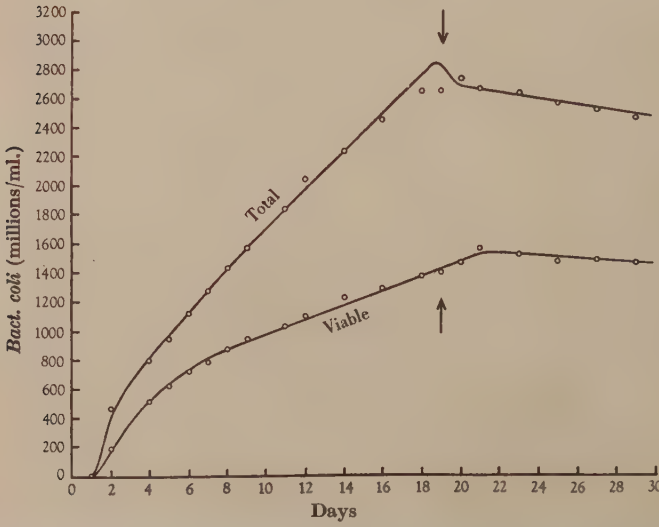

Fig. 4. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 20° and pH 6·0. The food supply was stopped shortly before taking sample marked by arrows.

The initial phase

It is highly probable that food, which was being added continually, at first accumulated, because the 300–400 cells/ml. of the inoculum were unable to use it as fast as it was supplied. Later, this accumulation would be used more or less rapidly according to conditions, until it was exhausted and the culture entirely dependent on the regular additions of food. The establishment of that state is held to mark the end of the initial phase.

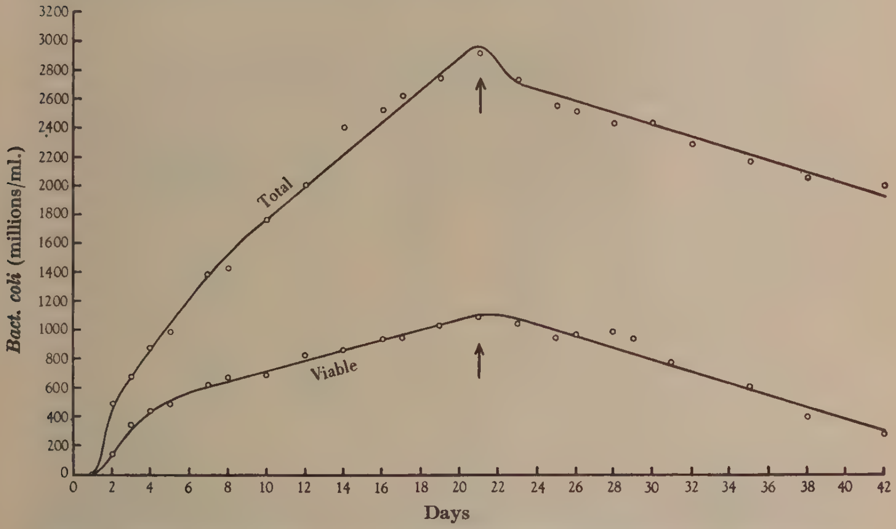

Fig. 5. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 20° and pH 9·0. The food supply was stopped shortly before taking sample marked by arrows.

Clearly, at both temperatures the commencement of development of the cultures at pH 5 was considerably delayed, but when growth did start it was exceedingly rapid, due presumably to the accumulation of a relatively large amount of food during the period of delay. This delay was longer at 20 than at 30°, and this was also evident at pH 6.

In Table 1 the duration of the initial phase, the total and viable cell populations at its termination, and the ratio of these values for each experiment, are given, together with the final cell yield in relation to the duration of the initial phase, i.e. in relation to the total food added during that phase. To complete the picture, data from the earlier experiments at pH 7 are included in this and subsequent Tables. The initial phase was longer at the lower temperature and the yield of cells greater both absolutely and relative to the phase length. This agrees with previous findings at pH 7 (Jordan & Jacobs, 1947). The duration of the initial phase tended to be shorter at the lower pH values, but the effect was slight, and it is noteworthy that the long delay in development at pH 5 had a barely perceptible influence on the duration of the initial phase. Also, the total and viable cell populations at the end of the phase varied but slightly, being on the whole smaller at the lower pH values. In consequence, the cell yields were, with one exception, directly proportional to the phase length. They depended directly on the amount of food added during the phase, and variations of pH within wide limits had no appreciable overall effect on the conversion of food into cells. The exception is the case of 20° and pH 9, where, despite a normal yield of total cells, that of viable cells was depressed, indicating a tendency for a larger proportion of the cells formed to be non-viable or to lose their viability readily.

Table 1. *The duration of the initial phase and the yield of cells under various conditions of* pH *and temperature*

Temp. (° C.)	pH	Duration of initial phase (days)	Yield of cells at end of initial phase (millions/ml.)		Ratio of total to viable count	Mean rate of cell formation (millions/ml./day)	
			Total	Viable		Total	Viable
20	5·0	7	1280	765	1·67	183	109
	6·0	7	1290	820	1·57	184	117
	7·0	8	1480	880	1·68	185	110
	9·0	8	1530	655	2·34	191	82
30	5·0	5	750	390	1·93	150	78
	6·0	4·5	650	315	2·06	144	70
	7·0	6	945	445	2·12	159	74

The constancy of the ratio of total to viable cells at a given temperature follows from the absence of any marked effect of pH on the cell yields. But whereas the ratios at 30° are all close to 2·0, with a slight tendency for the value to fall with increasing acidity, at 20° the values (apart from the exception noted) are lower and have a mean of 1·64 (Table 1). According to this evidence the value of the ratio would appear to be characteristic of the temperature and independent of the pH within certain limits.

The steady phase

The effect of pH on the behaviour of the cultures during the steady phase is clearly revealed by the values of the slopes of the calculated lines of regression of total and viable count on time (ΔT and ΔV respectively), which are given in Table 2. As anticipated from experiments at pH 7 previously reported, ΔT and ΔV were both larger at 20 than at 30° at pH 5 and 6. The effect of variation in pH on ΔT was slight in comparison with that of temperature, but quite definite, and clearly at both temperatures a low pH tended to enhance the yield of cell substance from a given amount of food. This effect was not apparent at the end of the initial phase, probably in part because of the shortness of that phase and in part because of the complicated conditions of food concentration then existing. Above neutrality an increase in pH had very little effect on ΔT, but below neutrality its increase with falling pH was approximately linear over the range studied. The effect of pH variation on

ΔV was generally similar to that on ΔT but rather more marked, and the alkaline reaction had a distinctly adverse influence.

The ratio $\Delta T_{20}/\Delta T_{30}$ is in the nature of a temperature coefficient, and it is noteworthy that its magnitude is practically independent of pH, the actual values being 1·87, 1·80 and 1·78 at pH 5, 6 and 7 respectively. The ratio $\Delta V_{20}/\Delta V_{30}$ is less constant, although two of the three values are very similar. They are respectively 2·11, 1·64 and 2·07. With increasing acidity, at both temperatures ΔV was actually enhanced in relation to ΔT. This aspect is best expressed in terms of the non-viability index (n.v.i.), previously defined as the proportion of the cells formed in a generation which is non-viable (Jordan & Jacobs, 1944, 1947). This index is obtained by calculating the quantity $\frac{1}{2}(1-\Delta V/\Delta T)$ and its values are also given in Table 2. With one irregularity at 20° and pH 6, the n.v.i. fell with diminishing pH, and thus the conclusion is reached that for the maximum yields of cell substance and of viable cells from a given amount of food, a low temperature and acid conditions are most favourable. This was not reflected in the yields of cells at the end of the initial phase, probably because the effect of acidity, though definite, was relatively slight and the time was too short for an observable effect to appear, except at pH 9. At this alkaline reaction the n.v.i. had the highest value of those recorded in the present series of experiments.

Table 2. *The effect of* pH *on the linear relation between numbers of total and viable cells and time in the steady phase, and on the non-viability index*

Temp. (° C.)	pH	Equation of regression of total count on time $T=T_s+\Delta T(t-t_s)$*	Standard error of ΔT	Equation of regression of viable count on time $V=V_s+\Delta V(t-t_s)$*	Standard error of ΔV	Non-viability index†
20	5·0	$T=1280+141{\cdot}2(t-7)$	±5·27	$V=765+75{\cdot}3(t-7)$	±5·71	0·233
	6·0	$1290+128{\cdot}6(t-7)$	±4·36	$820+50{\cdot}2(t-7)$	±1·68	0·305
	7·0	$1480+118{\cdot}9(t-8)$	±3·86	$880+50{\cdot}6(t-8)$	±2·23	0·287
	9·0	$1530+115{\cdot}2(t-8)$	±7·05	$655+33{\cdot}8(t-8)$	±1·31	0·354
30	5·0	$750+75{\cdot}7(t-5)$	±7·81	$390+35{\cdot}7(t-5)$	±3·70	0·264
	6·0	$650+71{\cdot}4(t-4{\cdot}5)$	±2·21	$315+30{\cdot}7(t-4{\cdot}5)$	±1·14	0·285
	7·0	$945+66{\cdot}8(t-6)$	±2·41	$445+24{\cdot}4(t-6)$	±3·28	0·318

* T_s = total cell count in millions/ml. at start of steady phase.
t = time in days.
t_s = time at which steady phase began.
ΔT = rate of increase in total count in millions/ml./day.
V_s = viable count in millions/ml. at start of steady phase.
ΔV = rate of increase in viable count in millions/ml./day.
† non-viability index $=\frac{1}{2}(1-\Delta V/\Delta T)$.

The starvation phase

Assessment on a simple basis of the behaviour of the cultures during starvation is difficult because the changes in turbidity and numbers of viable cells were not always regular nor consistently in one direction. There was a suggestion in some of the growth and death curves of a step-wise change, but the general trends are revealed if the starvation phase is treated in the same way as the steady phase, i.e. as if regular daily increments or decrements had occurred. The behaviour of each culture can then be summarized in terms of the slopes of the calculated lines of regression of cell numbers on time (Table 3). As a result of adopting this procedure it becomes evident from the figures that in all experiments except one, the apparent total cell count dropped suddenly immediately after the food supply was stopped. This is attributed to shrinkage of the cells in the changed environment, and implies that the factor for converting turbidities to cell numbers had altered.

Table 3. *The average daily changes in total cell count* (ΔT) *and viable count* (ΔV) *during starvation, at various values of* pH *and temperature*

Temp. (° C.)	pH	ΔT (millions/ml./day)	ΔV (millions/ml./day)
20	5·0	$-17{\cdot}9 \pm 10{\cdot}9$	$+\ 2{\cdot}1 \pm 2{\cdot}2$
	6·0	$-22{\cdot}6 \pm 3{\cdot}4$	$-11{\cdot}2 \pm 2{\cdot}6$
	9·0	$-40{\cdot}2 \pm 3{\cdot}5$	$-40{\cdot}3 \pm 4{\cdot}0$
30	5·0	$+21{\cdot}2 \pm 6{\cdot}1$	$+\ 4{\cdot}3 \pm 6{\cdot}1$
	6·0	$-13{\cdot}3 \pm 1{\cdot}8$	$-33{\cdot}8 \pm 1{\cdot}9$
	7·0	$-31{\cdot}5 \pm 3{\cdot}6$	$-22{\cdot}8 \pm 3{\cdot}5$

Apart from the case of 30° and pH 5, the apparent total cell count decreased during starvation, presumably as a result of autolytic processes leading to loss of opacity and disappearance of cells. These changes evidently became less rapid as acidity increased, and at the higher temperature. The latter finding agrees with results obtained at pH 7. Higher temperature may favour cell persistence by enhancing food storage by the cells, since from a given amount of food fewer cells are formed as the temperature is raised. The case of 30° and pH 5, where the total count continued to rise during starvation, is not really exceptional, since it accords with the general hypothesis that autolysis is reduced by acidity and higher temperature, but it would seem that considerable food storage must have occurred during the preceding phases to provide for the continued growth. This growth was balanced by death, since the viable count remained virtually constant, as it also did at 20°, pH 5. At pH 6, at both temperatures, the viable cell numbers continued to rise for a short time after the food was stopped, before a decline commenced. In these cases, therefore, the values of ΔV in Table 3 refer to the period following the rise. It appears that whereas the rate of fall was slow at 20°, a marked decline occurred at 30°, a result in accordance with that previously obtained at pH 7. At the only alkaline reaction employed the rate of decline was the highest of all. The picture is, on the whole, consistent, and it appears that the cells of these starved cultures survived best under acid conditions.

DISCUSSION

When the optimum pH and the limits of reaction for the growth of a bacterium are determined by the usual method, the presence of growth and its amount are judged by the degree of turbidity developed, so that total and not viable growth is the criterion by which the effect of pH is measured. Three factors are concerned in the growth of the cultures, namely, the duration of the lag phase, the rate of growth in the logarithmic phase, and the maximum population which the medium can support. Ordinarily no differentiation is made between these factors, but it would obviously be possible to determine the effect of changes in pH on all of them and to establish an optimum for each. These need not be identical.

Hinshelwood and his colleagues have established that the effect of pH on the growth of *Bact. lactis aerogenes* is exerted wholly on the level of the maximum population, there being no influence at all on the lag phase or the rate of growth (Hinshelwood, 1946). This result, if generally true, might lead to a definition of optimum pH as the value conducing to the maximum total cell population that a given medium can support. The growth curves of *Bact. coli* cultures supplied with food at a constant rate are, as shown above, very different from those grown under normal conditions of excess initial food supply. For instance, there is no evidence of the existence of a maximum population, and under both acid and alkaline conditions growth continued unchecked as long as food was supplied. The same was true of the viable cell numbers, and for these cultures the definition of optimum pH based on a maximum cell population is obviously meaningless. Constant growth rates, expressed in terms of the rates of increase in total and viable cells, did, however, exist, and they rose as pH was reduced. Actually, the maximum rates may not have been reached even at pH 5, the lowest value tested, but it seems probable that an optimum pH could be defined for these cultures as the value at which these rates were highest.

Cultures grown in the normal way with an initial excess of food may react to pH differently from those developing on a restricted but regular food supply. Shimwell (1935), working with *Lactobacillus pastorianus*, used acid production as the measure of growth. This probably depends on total rather than viable cell numbers, and this author also found that the growth rate was independent of pH. However, he was able to show that the lag phase increased as the pH was reduced. The results of the experiments described above suggest, although do not prove, that the lag phase of *Bact. coli* was increased at pH 5. Evidently the effect of pH on bacterial cultures is no simple one exerted invariably on a single phase of growth, but may vary from organism to organism and also with the method of cultivation.

It is clear that acidity, at least down to pH 5, increasingly favoured the growth of *Bact. coli* under these conditions of constant food supply, and also led to a greater viability among the cells formed. This behaviour is in striking contrast to the sensitivity to acidity of many of the enzymes of *Bact. coli*, as demonstrated by Gale & Epps (1942). These authors also showed that *Bact. coli* would grow in a casein digest between pH 4·5 and 9, and responded to changes in the external pH by alterations in the enzyme content of the cells, such that the external changes were counteracted and certain essential activities maintained at a constant level. No doubt the cells of cultures with constant food supply, when grown under acid or alkaline conditions, also responded in this manner, but it would be expected that towards the limits of the pH range the cells would be functioning with a somewhat reduced efficiency. Actually, near the lower limit of the range the cells were functioning more efficiently than at higher pH values, and this suggests that some other factor, as yet undiscovered, was predominant. The increased efficiency was shown not only in the higher growth rate and viability, but also apparently in the storage of reserve food, by virtue of which cell multiplication was able to continue for a considerable time after cessation of the food supply.

Previous results (Jordan & Jacobs, 1947) had shown that at pH 7 the ratio of total to viable cells at the close of the initial phase approximated to the value of 2·0 over a wide range of temperature. In the work reported above this ratio was also close to 2·0 at pH 6 and 5 when the temperature was 30°. However, at 20° the ratio at these pH values was lower (about 1·7), whereas at pH 9 it was higher (2·3). In the earlier work a value of 1·68 was obtained at 20° and pH 7 and was then regarded as a chance variation from the true value of 2·0, but since other similar values have now been obtained at that temperature under more acid conditions, and a value higher than 2·0 at an alkaline reaction, it is now considered that the value of 2·0 may be more susceptible to change with alteration of environmental conditions than was formerly thought to be the case.

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The Relation of Pantothenic Acid to Acetylcholine Formation by a Strain of *Lactobacillus plantarum*

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SUMMARY: Pantothenic acid was essential for the growth of a strain of *Lactobacillus plantarum*. It increased acetylcholine formation during growth and in washed suspensions of pantothenate-deficient organisms. Added pantothenic acid did not affect the glucose utilization or lactic acid formation by the pantothenate-deficient organism in washed suspension.

The co-enzyme concerned in acetylation of choline in brain and of sulphanilamide in liver has been shown to contain pantothenic acid (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1947). At the suggestion of Dr Lipmann, pantothenic acid was tested as a factor in the synthesis of acetylcholine by a strain of *Lactobacillus plantarum*. It proved to be essential both for acetylcholine formation and for growth (Stephenson & Rowatt, 1947). This paper contains a more detailed examination of the phenomenon.

Methods

The strain, provisionally identified as *L. plantarum*, was that described by Stephenson & Rowatt (1947), and it was maintained on media containing 1 % peptone instead of the tryptic digest of casein previously used. For washed suspensions, cells were grown on Stephenson & Rowatt's medium 5, biotin being omitted, as this had proved to have very little effect on growth. Pantothenate was not added.

Growth experiments were carried out in test-tubes each containing 10 ml. of the above medium with the inclusion of choline at a final concentration of 500 μg./ml. Calcium pantothenate, β-alanine, glucose, sodium acetate and cystein were sterilized by filtration. Pantoic lactone, kindly supplied by Dr F. Bergel of Roche Products Ltd., was sterilized and hydrolyzed by autoclaving in 0·1 N-NaOH. When deficiencies other than that of pantothenate were investigated, the casein used was an acid hydrolysate of vitamin-free casein supplied by Ashe Laboratories. The folic acid used was a concentrate supplied by Prof. F. C. Happold. For the effect of this substance on growth, synthetic pteroylglutamic acid was substituted. All cultures were incubated at 25° for 48 hr. unless otherwise stated.

Resting cells were resuspended in medium 6 of Stephenson & Rowatt (1947). Acetate was added and the glucose concentration used was 100 mg./ml. Calcium pantothenate and other compounds were added as stated. The suspensions were incubated at 25° for 3 or 4 hr.

Turbidity of suspensions and acetylcholine were estimated as previously described (Stephenson & Rowatt, 1947). Glucose was estimated by the method of Miller & van Slyke (1936) and lactic acid, after treatment with copper-lime, by the method of Friedemann & Graeser (1933).

Effect of pantothenic acid on growth

Cultures incubated for 24 hr. in a medium free from pantothenate showed no visible growth. A faint turbidity was apparent after 48 hr., but this did not increase on further incubation. In a medium containing pantothenate, growth was visible in 24 hr. and was almost complete after 48 hr.; maximal growth was obtained with 0·02 μg./ml. calcium pantothenate (Fig. 1).

Pantoic lactone or β-alanine (0·1 μg./ml.) did not increase growth on the pantothenate-deficient medium. If both were added together, growth was slightly greater at levels equivalent to 0·02, but not 0·01 μg./ml. calcium pantothenate (Table 1).

Table 1. *Substitution of β-alanine and pantoic lactone for pantothenic acid in media for growth of* Lactobacillus plantarum

Calcium pantothenate added (μg./ml. of medium)	—	0·1	—	—	—	—	—
β-alanine added (μg./ml. of medium)	—	—	0·1	—	0·004	0·008	0·1
Pantoic lactone added (μg./ml. of medium)	—	—	—	0·1	0·006	0·012	0·1
Dry weight of cells produced (mg./ml. of medium)	0·027	0·222	0·028	0·027	0·030	0·058	0·067
Acetylcholine formed (μg./ml. of medium)	0·44	4·8	0·36	0·34	0·34	0·48	0·50

Effect of pantothenic acid on acetylcholine production during growth

An increase in level of pantothenate in the growth medium caused an increase in the acetylcholine formed, whether this was calculated per unit volume of the medium or per unit dry weight of cells produced (Figs. 2, 3). Acetylcholine formation continued slowly for some days at pantothenate concentrations above 0·005 μg./ml., but at this level the acetylcholine content remained low. In the pantothenate-free medium, turbidity readings and acetylcholine values tended to be unreliable. This was especially so in the case of acetylcholine for which the values (per mg. cells) were very high, probably on account of substances present in the medium which increased the acetylcholine contraction of the rectus when present above a certain liminal concentration. At higher titres of acetylcholine, a smaller volume of medium was necessary for assay, and the liminal concentration of these substances was not reached. Pantoic lactone and β-alanine added separately again did not replace pantothenic acid and together gave only a small effect (Table 1).

Each of the growth factors present in the medium was omitted in turn. Nicotinamide was the only substance stimulating growth and, with it, acetylation of choline. Acetylcholine formation per mg. of cells was unaffected (Table 2(*a*)). *p*-Aminobenzoic acid and folic acid were treated together, both being left out of the medium and the effect of one in the absence of the other

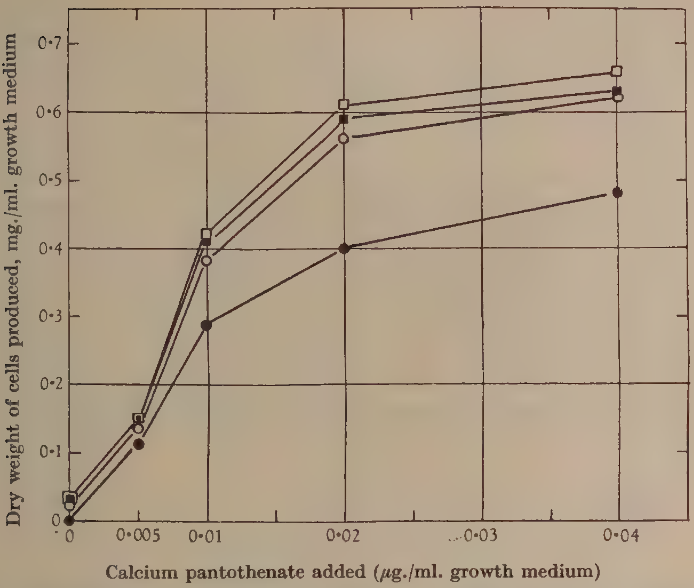

Fig. 1. The effect of pantothenic acid on growth of *L. plantarum* after incubation at 25° for 24 hr. ●——●; 48 hr. ○——○; 72 hr. ■——■; 120 hr. □——□. Growth is not visible at 24 hr. in absence of pantothenate and cannot be measured turbidimetrically.

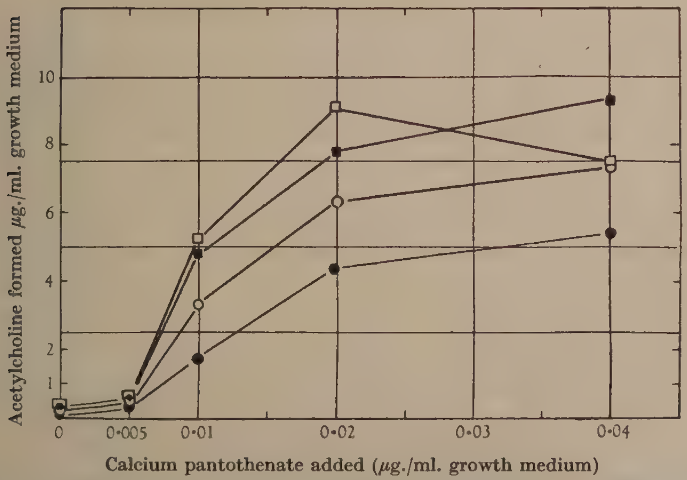

Fig. 2. The effect of pantothenic acid on acetylcholine formation per ml. of medium in growth of *L. plantarum* after incubation for 24 hr. ●——●; 48 hr. ○——○; 72 hr. ■——■; 120 hr. □——□.

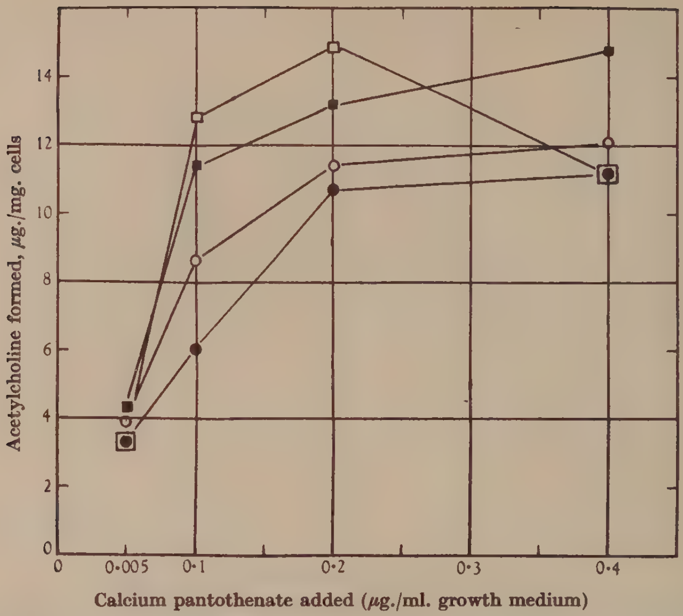

Fig. 3. The effect of pantothenic acid on acetylcholine formation per mg. cells in growth of *L. plantarum* after incubation for 24 hr. ●——●; 48 hr. ○——○; 72 hr. ■——■; 120 hr. □——□.

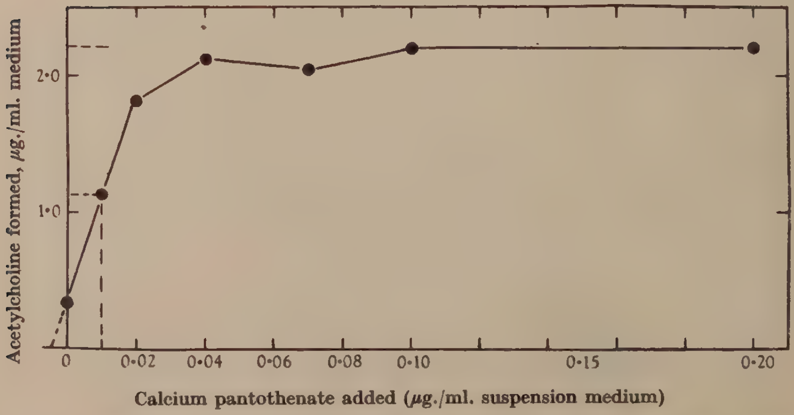

Fig. 4. The effect of pantothenic acid on acetylcholine formation by pantothenate-deficient cells of *L. plantarum* in washed suspension. The curve is produced back through the origin to cut the base, and by this means the total quantity of pantothenate in the medium is found.

determined. No effect was observed on growth, but each seemed to affect acetylcholine formation (Table 2 (*b*)). Woods & Nimmo Smith (1947) have found *p*-aminobenzoic acid to be a factor in the growth of this strain, suggesting that this substance was present in our medium. The effect of traces of growth factor carried over in inocula was not eliminated by serial subculture on the deficient media.

Table 2. *Effect of nicotinamide, p-aminobenzoic acid and folic acid on growth and acetylcholine formation of* Lactobacillus plantarum

(*a*)

Nicotinamide added (μg./ml. of medium)	0	2
Dry weight of cells produced (mg./ml. of medium)	0·185	0·290
Acetylcholine formed (μg./mg. cells)	25·9	23·1

(*b*)

Pteroylglutamic acid added (μg./ml. of medium)	0	0·01	0	0·01
p-Aminobenzoic acid added (μg./ml. of medium)	0	0	0·1	0·1
Dry weight of cells produced (mg./ml. of medium)	0·335	0·340	0·340	0·335
Acetylcholine formed (μg./mg. cells)	24·8	32·4	40	41

Effect of pantothenate on acetylcholine formation by pantothenate-deficient cells in washed suspension

The crop from a litre of medium deficient in pantothenate was very small, of the order of 10–20 mg./l. This contained very little acetylcholine initially, and incubation in medium 6 for 4 hr. caused an increase of as much as twice the original. With added pantothenate, a 5–6-fold increase was obtained. Under these conditions, the enzyme system became saturated with pantothenate by the addition of 0·04 μg./ml. calcium pantothenate to the suspension (Fig. 4). The dry weight of the cells was 0·58 mg./ml. From the curve shown in Fig. 4 it is seen that the concentration of calcium pantothenate giving 50 % saturation of the enzyme system is *c.* 0·014 μg./ml.

Addition of pantothenate to washed suspensions of cells grown in an optimal concentration of pantothenate did not increase acetylcholine formation. β-Alanine and pantoic lactone added at a level equivalent to 0·1 mg./ml. calcium pantothenate did not increase acetylcholine formation by pantothenate-deficient cells in washed suspension.

Although the addition of pantothenate to deficient suspensions increases acetylcholine formation, it had no effect on glucose utilization or lactic acid formation. For example, the glucose concentration of the suspending medium was reduced to 1·5 mg./ml., and the suspension, containing 0·66 mg./ml. cells, was incubated for 3 hr. Acetylcholine, glucose and lactic acid were estimated before and after incubation. In absence of added pantothenate, utilization of 510 μg. glucose gave rise to 394 μg. lactic acid, while 0·03 μg. acetylcholine was formed. When pantothenate was added, 490 μg. glucose was fermented to give 392 μg. lactic acid with the formation of 1·6 μg. acetylcholine. The percentage of lactic acid formed from glucose by normal suspensions of this organism is of the same order (Stephenson & Rowatt, 1947).

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The Serology of *Pseudomonas pyocyanea*

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SUMMARY: Three main antigens were found to be variously distributed amongst strains of *Pseudomonas pyocyanea*. The serological behaviour of the strains studied depended on the presence and quantity of these antigens. It was not possible to distinguish between flagellar and somatic antigens, but those found were probably somatic.

Amongst the peculiarities of serological behaviour of bacteria in this species the most striking was a complete loss of agglutinability on moderate heating, which left the antigenic activity unimpaired.

The classification of the genus *Pseudomonas* is, like most bacteriological classifications, one of contradictions. The species *Ps. pyocyanea* is not clearly delimited and most observers have found strains which, though most conveniently placed in that species, had one or more atypical characteristics. Within the species, classification has been attempted, but without conspicuous success. Gessard (1919, 1920), who made the first extensive study of the organism, based his classification entirely on pigment production. On this basis he not only determined whether a strain should be included in the species, but divided it into three varieties, each again subdivided into four 'races'. The validity of this classification depends on the assumption that pigment production is a constant property of strains; and ordinary laboratory experience does not support that assumption.

Aoki (1926) attempted a classification on the basis of cultural and serological characteristics. He found great variations in the association of different cultural characteristics, and treated *Ps. pyocyanea*, *Ps. fluorescens* and *Ps. putida* as one species, since a large number of strains could not be placed definitely in one or the other of these three previously described species. He found 22 serological types among 50 strains, and here again a clear division into three species was not possible. From his tables, however, it appears that each serological group contained only such strains as did not differ in pigment production. Sandiford (1937) tested 50 strains and also concluded that no cultural or serological distinction between *Ps. pyocyanea* and *Ps. fluorescens* was possible. Trommsdorff (1916) similarly investigated organisms of all three species and found that the results of cultural and serological classification did not coincide. By agglutination he could divide 25 out of 27 *pyocyanea* strains into three groups. He obtained cross-agglutination reactions between the three species, but concluded on the basis of absorption experiments that the three species were not antigenically identical.

Knowledge of bacterial dissociation has had few repercussions in the *Pseudomonas* group, and it is still doubtful whether an $S \rightarrow R$ variation occurs. Recently Gaby (1946) described three basic colony variants that were present in each of 12 strains of *Ps. pyocyanea*. These variants were not stable, each giving rise on subculture to the other variants. In 10 of the 12 strains the three variants were serologically different, and the analogous variants of various strains serologically identical. The remaining two were not agglutinated by any of Gaby's type sera. It is probable, therefore, that Gaby investigated the variants of one serological group only. The serological differences between the variants Gaby assumes to be due to differences in flagellar antigens, whereas the somatic antigens are identical. This conclusion is based on the agglutination results with formalin- and ethanol-treated suspensions. From his tables it is apparent that the differences between the agglutination of formalin-treated suspensions of the three variants are clear-cut and his conclusions as to the identity of the corresponding variants in all strains tested are justified. The agglutination titres with ethanol-treated suspensions are more or less uniformly low.

Brutsaert (1924) investigated the serological relationships between O and H antigens in this group, using mainly one strain, on lines similar to those followed generally in working with H and O antigens in the Salmonella group. His 'complete H and O antigens' was a suspension heated 1 hr. at 56°, and his O antigen a suspension boiled for 2 hr. Antisera prepared with the first suspension agglutinated only 'H+O' suspensions; the serum prepared with the boiled suspension agglutinated both kinds of suspension, as did also a serum prepared with a boiled O-variant of another *pyocyanea* strain.

Experimental

Strains growing in blue-green colonies with iridescent spots, and matt yellow strains, thus widely different in their cultural characteristics, were selected on the assumption they would differ also in antigenic structure. The first two sera were accordingly prepared against a blue iridescent and a matt yellow strain respectively. The sera, however, gave strong cross-reactions, and agglutinated 60 strains of *Ps. pyocyanea* to approximately the same titre. With cross-absorption tests only minor differences in the antigenic structure of the two strains were found. Other sera were prepared with strains not agglutinated by these sera, until finally five sera were available, one of them, no. IV, being prepared against a non-motile strain for serological analysis of the organism.

All these sera were prepared by injecting rabbits 5–6 times at weekly intervals with suspensions heated for $\frac{1}{2}$ hr. at 60°, the doses rising from 20 to 1000 million organisms. The strains tested were from different sources, most of them from human material like pus (from various sites), sputum, faeces, cervix uteri, and cerebrospinal fluid; very few came from water. Those which did not grow at 37° were excluded on the grounds that they were unlikely to be true *Ps. pyocyanea*. The agglutinations were carried out with the unheated living suspensions, first at 37° for 2–4 hr., then at room temperature overnight. The agglutination was O in type.

In Table 1 the patterns of agglutination and the number of strains producing them are set out. It would appear that there are three main antigens, A, B and C, for which the corresponding antibodies are contained in sera II, IV and V respectively; and that all the possible patterns of agglutination depend, generally speaking, on the presence or absence or else the difference in quantity of these three antigens. The 54 strains may be grouped as follows.

Mainly A	antigen	28	strains
,, A+B	,,	8	,,
,, B	,,	4	,,
,, C	,,	5	,,
,, B+C	,,	1	,,
Small amounts of one or the other antigen		6	,,
Not agglutinated		2	,,

It is possible that the five strains which agglutinate only to low titre with sera I and IV have another main antigen in common, which may be present also in those two B strains which agglutinated to a low titre with serum I.

Table 1. *Serological relations of 54 strains of* Pseudomonas pyocyanea

Suspensions of living organisms tested		Sera and predominating antibody				
Predominant serological type	No. of strains	I A	II A	III A+B	IV B	V C
		Observed agglutination				
A	8	+++	+++	+++	−	−
	3	+++	+++	+++	±	−
	15	+++	+++	+++	+	−
	1	+++	+++	+++	−	+
	1	+++	+++	+++	±	+
A and B	6	+++	+++	+++	+++	−
	2	+++	++	+++	+++	±
C	5	+	−	−	+	+++
B	2	+	−	+	+++	−
	2	−	−	++	+++	±
B and C	1	−	−	+	+++	+++
Miscellaneous	1	+	±	±	−	−
	1	+	−	−	±	−
	2	+	−	−	+	−
	1	+	−	−	±	±
	1	±	−	−	+	+
	2	−	−	−	−	±

± = partial agglutination to 1/160.
+, ++ and +++ = complete or almost complete agglutination to 1/160, 1/640 and 1/1280 or more, respectively.

Numerous absorption experiments were carried out between the groups A, A+B and B. They did not bring any new facts to light, but confirmed only the conclusions drawn on the basis of direct agglutinations. Serum III contains predominantly A-antibodies and only a small quantity of B-antibodies; the strain, however, with which it was prepared was agglutinated to titre by an anti-B serum, and absorption of serum III with an A strain left the B agglutinins unaffected. Some absorption experiments carried out within group A and within group B showed that within the group the strains were not completely identical; minor differences were present. Each group contained blue as well as yellow strains excepting group B in which all were yellow. Of the 54 strains three were non-motile; two in group B, and one, a blue strain, contained only small amounts of all three antigens.

In the above analysis there is no distinction of somatic and flagellar antigens. By analogy with the heat-labile flagellar antigens of the *Salmonella* and *Proteus* genera, antisera were prepared against bacterial suspensions heated in a boiling water bath for 2 hr. The strains were the same as those used to prepare sera I and II, namely P8 and 1602. Agglutination tests were made with living suspensions.

The antiserum to boiled P8 had lower titres throughout as compared with those of corresponding serum I; with some strains the drop was considerably greater than with others. The titres with the antiserum to boiled 1602 were identical with those of the corresponding serum II. The results of these experiments suggest that there is no more than a quantitative difference between the antisera obtained with the two differently treated suspensions.

An attempt to make a serum rich in flagellar antibodies was made by the method of Gohar (1932) for the preparation of *Vibrio cholerae* H antisera. A bacterial suspension of strain 'Molyneux' was shaken to tear the flagella off and centrifuged at moderate speed. The supernatant fluid, supposed rich in flagella, was then added to a bacterial suspension containing the full antigen, and the mixture used for immunization. A very high titre was obtained, but this serum again showed much the same range as the corresponding serum III. The appearance of the agglutination was definitely of the granular O type in both sera. Both serum III and the corresponding III flagellar serum were absorbed with suspensions of normal organisms, of ethanol-treated organisms or of organisms grown on phenol agar. But the results were never quite clear-cut enough to warrant drawing definite conclusions.

Having thus failed to obtain any conclusive evidence for the presence of distinct O and H antigens by this method, sera prepared against the whole untreated organism were tested against bacterial suspensions treated so as to serve as indicators for H and O agglutinins.

The usual procedure was adopted of treating bacterial suspensions with formalin for the preservation of H antigens in bacterial suspensions, and either with ethanol or heat at 100° to obtain O suspensions. Sixteen strains were chosen; three predominantly A, seven predominantly A + B, two predominantly B, one C, one B + C and two strains which did not show any appreciable agglutination with any of the type sera, but which might manifest masked antigens as a result of the treatment. Sera I, II, III, IV, V and III flagellar were tested (Table 2).

The agglutinations with the formalin-treated suspensions were straight forward enough. In serum II, the purest A-serum available, the formalinized A strains were agglutinated to titre, but the A + B strains had lost a good deal of their agglutinability. In serum I, also an A-serum, only two of the three A strains were clearly agglutinated. The third, and the A + B strains, showed an appreciable loss of agglutinability. Serum III and serum III flagellar

Table 2. *Agglutination of formalinized (F) and of ethanol-treated (E) suspensions of* Pseudomonas pyocyanea

The figures give the titre to which there was complete or almost complete agglutination.
+ = slight agglutination to a titre of 640 or higher.
± = slight agglutination to a low titre or trace of agglutination to any titre.
The strains P 8, 1602, Molyneux, J and Lewis were used to prepare sera I, II, III, IV and V respectively; the figures and symbols in heavy type record the agglutination of strains by homologous antisera.

		Sera											
		Anti-A I		Anti-A II		Anti-A+P III		Anti A+B III flagellar		Anti-B IV		Anti-C V	
Strain	Antigenic group	F	E	F	E	F	E	F	E	F	E	F	E
Br. S. 179	A	640	+	640	±	±	–	±	–	–	–	–	–
P 8	A	**1280**	**320**	640	320	+	–	+	–	–	–	–	–
1602	A	±	–	**1280**	**–**	±	–	+	–	–	–	–	–
Molyneux	A+B	+	–	+	–	**2560**	**+**	2560	+	640	–	±	–
Cervix	A+B	±	–	+	–	+	+	1280	640	640	+	–	–
Faec. 2936	A+B	+	–	+	–	1280	+	2560	1280	640	±	–	–
Faec. 2433	A+B	±	–	+	–	1280	–	2560	–	1280	–	–	–
Sm blue	A+B	+	–	+	–	1280	320	2560	640	640	–	–	–
Z	A+B	±	–	±	–	1280	80	2560	+	1280	160	±	–
Path	A+B	320	–	+	–	640	320	2560	160	640	+	±	–
Emery	B motile	±	–	–	–	±	–	320	–	320	–	–	–
Faec. 3963	Not definite	–	–	–	–	–	–	–	–	–	–	–	–
Faec. 4656	Not definite, non-motile	–	–	–	–	–	–	+	–	–	–	–	–
Lewis	C	–	–	–	–	–	–	80	–	–	–	**1280**	**–**
470	B+C	–	–	–	–	320	320	640	320	640	640	5120	–
J	B non-motile	±	–	–	–	320	320	1280	640	**1280**	**1280**	±	–

agglutinated the A + B strains, B + C strain and one of the B strains, the other B strain showed only traces of agglutination by serum III, but a clear-cut reaction with serum III flagellar; the agglutination of formalinized A strains was markedly diminished. The B serum and C serum agglutinated the formalinized suspensions to the same range and titre as the living suspensions.

The agglutination of formalinized suspensions only confirmed the grouping arrived at by the agglutination reactions with living organisms. Antigen A was affected by the formalin treatment to a considerable degree. By analogy with the Salmonellae, the B antigen, the main somatic antigen of two non-motile strains, should have been affected at least to the same degree.

The agglutination of formalinized flagellated organisms by a serum like IV which contains probably only antibodies against somatic antigens would not occur in peritrichously flagellated organisms like those of the Salmonella group. That such an agglutination takes place with formalinized *Ps. pyocyanea* strains suggests that the antigenic modification by formalin of organisms with one terminal flagellum is not analogous with that of the Salmonellae.

In the preparation of ethanol suspensions it was found that suspensions kept in 30 % (v/v) ethanol-water lost their agglutinability within 2 days. Suspensions were therefore prepared by holding the bacteria in absolute ethanol at 45° for 1 hr., at room temperature for 2 hr., then centrifuging and resuspending them in saline. Various attempts were made to grow the organisms for these suspensions on phenol agar to diminish flagellation, but even very small amounts of phenol decreased the yield largely and the concentration of phenol finally adopted, 1:2000, affected neither the growth nor the motility of the bacteria.

Agglutinations with ethanol-treated suspensions gave very much the same result as those with formalinized suspensions. The titre was lowered; and a few strains had lost their agglutinability altogether. For instance, the strain used to prepare serum II was not agglutinated by its own serum though strain I still was. One of the B strains was similarly inagglutinable. Strain 2433, an A + B strain, also lost its agglutinability after ethanol treatment, whereas in formalinized suspension it was agglutinated to titre by serum IV, prepared with a non-motile B strain. As already mentioned, the formalinized suspensions of all A + B strains were agglutinated to titre by an anti-B serum, though from an orthodox viewpoint one might expect only the flagella in these formalinized suspensions to be reactive and no antibodies for them to be present in the B serum. Serum V, a pure C serum, while agglutinating the C strain and the B + C strain in formalinized suspensions, did not affect the ethanol-treated suspensions. Serum III, prepared with an A + B strain, agglutinated the ethanol suspensions of one strain B, and the B + C strain to titre, but its agglutination with A + B strains was much reduced. The A antigen, which had seemed to suffer most from formalin treatment, showed a still heavier loss in ethanol suspensions. There was not one instance in which the ethanol treatment had preserved an agglutinability which the formalin had destroyed.

Tests with heated suspensions were made, but had to be abandoned in view of the discovery that suspensions of *Ps. pyocyanea* lost their agglutinability completely after heating. This loss occurred in suspensions heated 2 hr. in a boiling water bath, but was just as complete in suspensions heated for ½ hr. at 60° and was quite marked in suspensions heated for ½ hr. at 53°. This is the more remarkable since all the sera had been prepared with suspensions heated at 60° for half an hour. This is to say, with suspensions not themselves agglutinable it had been possible to produce sera of very high titre.

DISCUSSION

The experimental results described provide evidence for the existence in the species *Ps. pyocyanea* of three or possibly four main antigens. These antigens occur alone or in various combinations. It has not been possible to assign these antigens to definite parts of the bacterial surface, body or flagella. The reason for this failure is probably as follows. Of the usual methods for preparing O agglutinated suspensions heating must be excluded, as it makes the organisms inagglutinable, though they are still antigenic. A somewhat similar phenomenon has been described by Bawden & Kleczkowski (1941, 1942), who found that certain antigens, when heated in the presence of other proteins, could no longer be precipitated by anti-serum though they were still antigenic; if heated alone, however, they retained their reactivity. The *pyocyanea* suspensions had been heated in pure salt solution, but it is possible that internal rearrangements in the chemically complex bacterial body produced combinations of antigenic and non-antigenic components such as Bawden & Kleczkowski obtained by adding other proteins to their specific antigens. This phenomenon is being investigated further.

The ethanol treatment of organisms of this group appears to result in an impairment of the agglutinability, greater in degree than that caused by formalin treatment, but not differing from it in the distribution amongst various strains, so as to allow distinguishing between flagellar and somatic antigens. Some ethanol-treated suspensions were no longer agglutinated even by the homologous antiserum; and some ethanol-treated suspensions of strains containing B antigen were no longer agglutinated by serum IV which, being prepared with a non-flagellated strain, probably contains mainly somatic antibodies. In formalinized suspensions, on the other hand, the flagellated organisms containing antigen B were still agglutinated to titre by an O-serum against B; this is to say, they behave like formalinized non-flagellated organisms.

The formalinization of a non-flagellated organism of any kind does not reduce its O-agglutinability. It is only in flagellated organisms that the formalin-fixed flagella prevent access of antibodies to the surface of the bacterial body. This is understandable with organisms that are peritrichously flagellated. But it will not necessarily follow for an organism with monotrichate flagellation. Gaby seems to have been the first to test formalinized and ethanol-treated suspensions of monotrichate organisms and to assume that the results so obtained had the same significance as in *Proteus* and *Salmonella*.

Balteanu (1926), who analysed the antigenic structure of the cholera *vibrio*, used only the thermolability of the H and thermostability of the O antigen to distinguish between the two; and so did Brutsaert in his attempt to distinguish H and O antigens in *Ps. pyocyanea.* Gaby's absorption experiments with formalinized cells are beautifully clear-cut, and there is no doubt that he has revealed definite serological differences between the variant types. The variants clearly have one antigen in common which he assumes to be somatic, and differ in another antigen which he assumes to be flagella. But in the light of the demonstration here that agglutinability may be markedly diminished by a short ethanol treatment, further details of his ethanol treatment of cell suspensions are needed. Furthermore, one would hesitate to assume that the somatic antigen is invariably preserved and the flagellar antigen destroyed by ethanol treatment.

With regard to the preparation of agglutinating antisera all attempts to prepare antisera with distinct O and H antibodies for organisms of this group have failed. Brutsaert showed that a serum prepared with an organism heated at 56° agglutinates such a heated suspension, but not one held for 2 hr. at 100°. So far this would correspond with the present experience that a heated suspension becomes inagglutinable; but his report of agglutination of both suspensions by a serum prepared with boiled organisms is wholly at variance with my results, unless he was dealing with an exceptional strain.

The use of bacteriophages for exploring antigenic structure promises well in this group. Preliminary experiments show that two phages, isolated by using an A strain and a B strain respectively as indicators, almost completely corresponded with the serological grouping in their attack on the various strains. The A strains were attacked by the A phage, the B strains by the B phage, the A+B strains by both; the C strains were attacked only by the A phage, the B+C strain again by both phages. As a rule phage susceptibility depends on somatic antigens, though Sertic & Boulgakov (1936) have described a typhoid phage whose action depends on the presence of a particular flagellar antigen.

My phage experiments strongly support the grouping arrived at by serological methods, and while they do not prove conclusively that all the antigens established in the above experiments are somatic antigens, they make it very probable.

The H antigens may be undetectable because they are small in amount compared with the somatic antigens or, more likely, because the type of flagellation and other peculiarities of the species make the methods used generally for the detection of H antigens entirely inadequate in *Ps. pyocyanea.* It is also possible that the flagellar antigen is not different from the somatic one, and that in antigenic structure the flagellum constitutes just a part of the surface of the bacterial body. This has not been demonstrated in any other micro-organism, but is not improbable. In this connexion Pijper's (1946) contention that the bacterial structures hitherto regarded as flagella are only deformations of the slimy surface layer of bacteria is clearly relevant.

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The Biological Assay of Streptomycin by a Modified Cylinder Plate Method

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SUMMARY: The use of plate glass sheets carrying twenty-four cylinders in place of Petri dishes for the biological assay of streptomycin by the cylinder-plate method initially led to large assay errors. The substitution of cavities for cylinders, of a single layer of agar for two layers, one sterile and one inoculated, of the four-point assay design for 'reading off the curve', and of an optical projection method of measurement diminished the fiducial limits of error of an assay to 80–125% ($P=0{\cdot}95$).

Over the range of concentrations 0·1–100·0 units streptomycin/ml. the deviations from linearity of the graph of zone diameter against the logarithm of the concentration were very small.

Variations in thickness of the agar layer and in the intensity of inoculation had a marked effect on zone diameter.

An 8 × 8 Latin square lay-out reduced the internal error of an assay to 91–110% ($P=0{\cdot}95$), and with this increased accuracy a bias in the results was revealed arising from the time interval between filling successive cavities on the plate. This time effect was eliminated by the adoption of an appropriate order of filling, with a quasi-Latin square lay-out. The form of the assay finally adopted had fiducial limits of error, estimated both internally and externally, of about 95–105% ($P=0{\cdot}95$).

During the 1939–45 War it became increasingly difficult to procure adequate supplies of Petri dishes for the assay of penicillin on the well-known cylinder plate, and attempts were therefore made to use plate-glass sheets carried on wooden or aluminium frames as substitutes for the dishes. These attempts proved quite successful for plant control.

Wider variations than those permissible for research purposes, obtained when these frames were used for the biological assay of streptomycin, necessitated a closer examination of the factors which might influence the accuracy of the result. This paper is concerned solely with certain features which were found to influence the assay as carried out when using frames.

Initial techniques

The frames were either of hardwood or aluminium, the latter being the more desirable because they do not warp. Even so, it is somewhat difficult to get a frame which does not leak slightly, and because of possible variations in the amount of agar left on a plate with consequent variation in the size of the zones of inhibition, each plate was made a self-contained assay, thus avoiding reference of an unknown to a standard on another plate.

In early work the assays were made on plate-glass sheets, 32 × 14 cm., held in a frame whose inside measurements were 27½ × 10 cm. The nutrient agar was approximately 2 mm. deep and supported twenty-four porcelain cylinders of the well-known type. The twenty-four cylinders were arranged in three staggered rows of eight according to Table 1, where S_H represents the cylinders carrying the standard of higher concentration (40 units/ml.), S_L those carrying the standard of lower concentration (4 units/ml.), and U_1 and U_2 represent cylinders carrying solutions of unknown concentration. The plates were sterilized by dry heat at 160°, allowed to cool to about 80–90° and filled first with 60 ml. of sterile nutrient agar, and as soon as it was set, by 60 ml. of nutrient agar at 60° previously inoculated with *Bacillus subtilis*. As each layer of agar was poured the plate was placed on a level bench covered with plate glass, so that a double layer of agar of uniform thickness resulted.

Table 1. *Plate lay-out for two unknown solutions using rectangular plates and 'reading off the curve'*

S_L	U_1	U_2	S_H	U_2	S_L	S_H	U_1
S_H	S_L	U_1	U_2	U_1	U_2	S_L	S_H
U_2	S_H	S_L	U_1	S_H	U_1	U_2	S_L

The porcelain cylinders were sterilized by heating on a hot plate, picked up with sterile tweezers and dropped from a height of a few mm. on to the agar in the desired positions. The cylinders were then filled with the various solutions; the following precautions being taken:

(*a*) One person filled the cylinders on a given plate.

(*b*) The same filler (of the fountain-pen filler type) was used for all solutions.

(*c*) The same number of drops was used in each case.

(*d*) As far as possible the tip of the filler was held at the same height above the cylinders, to prevent the drop from touching the cylinders before becoming detached and to avoid the formation of 'air-bells', and the filler was held at the same angle (nearly vertical).

The potencies of the unknown solutions were estimated in the usual manner by 'reading off the curve'. Such precautions, amongst others, are commonly believed to lead to good assay results, but a check on the method, all operators being aware that it was being made, showed that twenty assays on the same sample could give results as widely discrepant as 5·8 and 19·5 units/ml.

Modifications in technique

(*a*) *The use of cavities in place of cylinders.* A number of features of the use of cylinders was considered undesirable, in particular, their variation in size and the variation in the depth to which they penetrated the agar. Cylinders which penetrated deeply into the agar would reduce the area of the zone through which the streptomycin solution would initially diffuse. Accordingly cavities were tried.

The plate was poured and allowed to set. After about 30 min. at room temperature the cavities were bored in the agar with a sterile no. 5 cork borer which was clean, sharp and free from burred edges. The agar disks were then removed with small sterilized spear-shaped needles. These were better than plain needles because the disks, once impaled, do not slip off, and a number can be impaled one after the other.

At first the test solutions crept between the agar and the glass plate, but this trouble was not encountered later.

For ready calculation of the internal errors of the assays the standard four-point assay method was used (e.g. Finney, 1944). In this the standard is used at two dilutions, and the unknown is also used at two dilutions, the dilution ratio being the same for both standard and unknown. The same lay-out as in Table 1 was used, with the modification that each plate carried only one unknown, and U_1 and U_2 represented its upper and lower dilutions.

A rough experiment showed that the accuracy with cavities was certainly no worse than that obtained with cylinders and was possibly better, so cavities were adopted forthwith.

(*b*) *The use of a single layer of agar.* It was found more convenient to pour the agar already inoculated on to the plate in one layer. This tended to produce a double edge to the zones of inhibition (not to be confused with a 'halo') for reasons which are not clear. The larger zone is on the upper surface of the agar, the smaller zone at the glass-agar interface. Experiment showed, however, that provided one or the other was chosen for measurement and the choice maintained there was no difference in the potencies obtained or in the standard error of their determinations.

(*c*) *Method of measurement of diameters of zones of inhibition.* At first, measurements of zone diameter were made with calipers, transparent rulers or a travelling microscope. The first two methods are not accurate enough and were, by comparison with the method now used, time-consuming and laborious. The travelling microscope was accurate but even more irksome to use. In our present apparatus, the zones are magnified approximately seven times without loss of definition, and the readings can be made almost as fast as they can be written down. Illumination is from a photoflood lamp in the top portion of the apparatus, and the light passes downwards through a condenser lens about 6 in. in diameter and of 3 in. focal length. The plates are placed beneath the lens and the transmitted beam then passes on to a 9 in. focal length anastigmatic portrait lens arranged below. At the bottom of the apparatus is a 6 × 6 in. mirror slightly tilted so as to throw the reflected image slightly forward and upwards on to the ground-glass screen. The apparatus may be extemporized from an ordinary projection lantern arranged vertically, the plates being inserted in the position normally occupied by the lantern slides. In all the experiments described below zone-diameter measurements are those obtained on such apparatus. Typical magnified zones have diameters of 10–20 cm., and the edges are quite sharp. These modifications reduced the 95 % fiducial limits of error of an assay to about 80–125 %.

The bases of the assay

(*a*) *The linearity of relationship between zone diameter and logarithm of concentration.* The linearity of the relationship between zone diameter and the logarithm of the concentration was tested on twenty plates each containing

60 ml. of agar and carrying twenty-four cavities. On each plate four cavities were filled with each of six solutions containing 0·1, 0·25, 0·5, 1·0, 10·0 and 100·0 units of streptomycin sulphate/ml. in pH 8·0 buffer. The average zone diameters are given in Table 2. The best straight line and the best parabola were fitted to the points at 0·1, 1·0, 10·0 and 100·0. The linear coefficient for the parabola was 3·659 ± 0·044, and the quadratic coefficient 0·0796 ± 0·0492. The quadratic coefficient thus cannot be considered significant ($P = 0{\cdot}1$). It is clear that the curvature over this wide range of concentration, even if it does exist, is nevertheless so small as to be of no consequence for practical purposes. The values for the two end-points (0·1 and 100·0) given by the best straight line and the best parabola have been inserted in Table 2 to show the smallness of the difference between the fit of the two curves.

Table 2. *Relation between zone diameter (cm.*) and logarithm of concentration*

	Concentration (units/ml.)					
	0·1	0·25	0·5	1·0	10·0	100·0
Observed average zone diameter*	11·38	12·78	13·99	15·30	18·81	22·41
Value calculated from best straight line	11·484	—	—	—	—	22·461
Value calculated from best parabola	11·405	—	—	—	—	22·382

* The measurements refer to the zone diameters on the screen of the measuring apparatus, which magnified about seven times.

(*b*) *Effect of uniformity of the agar layer.* The 95 % fiducial limits for the assay as carried out with the modification outlined above were approximately 80–125 %. This, though a substantial improvement on the earlier figures, was not good enough.

A factorial experiment was made to determine the effects of variation in the thickness of the agar and density of inoculation. The quantity of agar used was at two levels, normal and three times the normal, and the intensity of inoculation was at two levels, normal and four times the normal. Two plates were used at each of the four combinations of quantity of agar and intensity of inoculation, and three concentrations of streptomycin, 1, 5 and 10 units/ml., tested on each plate. The average diameters of the zones of inhibition are given in Table 3.

Table 3. *Average diameter of zones (cm.) of inhibition for different intensities of inoculation and quantities of agar*

	Intensity of inoculation								
	Normal Streptomycin units/ml.				Quadruple Streptomycin units/ml.				
	1	5	10	Average	1	5	10	Average	Average
Quantity of agar:									
Normal	15·48	16·92	17·85	16·75	14·10	15·29	16·16	15·18	15·96
Treble	14·01	15·29	16·18	15·16	12·01	12·96	13·96	12·98	14·07
Average				15·95				14·08	

To test the significance of these results we need the appropriate analysis of variance. This is rather unusual, as the error term for testing the effects involving the concentration is different from and much smaller in magnitude than that for testing the main effects of intensity of inoculation and quantity of agar and their mutual interaction (Table 4).

Table 4. *Analysis of variance of experiment on variation in quantity of agar and intensity of inoculation*

Source of variance	Degrees of freedom	Sums of squares	Mean squares	P
Intensity of inoculation (I)	1	2147·042	2147·042	0·05
Quantity of agar (Q)	1	2147·042	2147·042	0·05
Interaction of I and Q	1	57·042	57·042	$>0{\cdot}05$
Error term for I, Q and IQ	4	101·832	25·458	—
Concentration of solution (C)	2	1838·384	919·292	$<0{\cdot}001$
Interaction of C with I	2	8·583	4·292	$>0{\cdot}05$
Interaction of C with Q	2	4·083	2·042	$>0{\cdot}05$
Interaction of C with IQ	2	1·083	0·542	$>0{\cdot}05$
Error term for above four terms involving C	6	7·918	1·320	—
Total	21	6314·950		

The concentration of solution effect (C) is of course significant, but it is interesting to note that its interactions with I (intensity of inoculation) and Q (quantity of agar) are completely non-significant. That is to say, the slope (the increase in zone diameter with the logarithm of the concentration) is not significantly different for the two levels of intensity of inoculation and for the two levels of quantity of agar.

Both the intensity of inoculation and quantity of agar effects are highly significant, but their interaction is not significant. We can therefore conclude that they operate independently. Their magnitudes for the changes concerned, namely, four-fold for intensity of inoculation and three-fold for quantity of agar, are approximately the same, nearly 2 cm. on an average of 15 cm.

The Latin square and quasi-Latin square lay-out

The Latin square principle. From the experiment described in the previous section it was apparent that one possible source of error lay in the variations in the thickness of the agar; variations in the intensity of inoculation should of course not occur when working with a single layer of agar. Although the lay-out given in Table 1 was an attempt to give a balanced arrangement, plates of this shape will not accommodate a lay-out which would allow the effects of inhomogeneity in the agar to be removed from the error. Plates 35 × 35 cm. with aluminium frames 27½ × 27½ cm. (inside measurement) were constructed to accommodate sixty-four cavities laid out in the form of an 8 × 8 square. Each row contained solutions S_H, S_L, 1_H, 1_L, 2_H, 2_L, 3_H, 3_L, where S is the standard and 1, 2 and 3 are three unknown solutions, and the suffixes H and L denote the higher and lower concentration, a ratio of 10 to 1 being employed throughout. The use of the Latin square arrangement (Fisher,

1942) made it possible to have all eight treatments occurring once and only once in each row and also once and only once in each column. Differences between columns were thus independent of differences between rows and between treatments, differences between rows were independent of differences between columns and treatments, and, most important, differences between treatments were independent of differences between rows and between columns. Thus effects due to one or more rows and columns having agar different in thickness from the others did not introduce any error into the determination of the average zone diameters for S_H, S_L, 1_H, etc.

The use of this Latin square had the following general advantages over the earlier methods:

(*a*) An assay of three unknowns required sixty-four cavities instead of seventy-two with the earlier rectangular plates. Even with this decrease in total number of cavities, the number of observations at each point actually increased to eight instead of six as on the smaller plates.

(*b*) It was as easy and the plates took no longer to pour than smaller ones. Washing also took very little longer.

(*c*) The internal error of an assay was much reduced, the 95 % fiducial limits being about 91 and 110 %. These limits being rather less than half those of the earlier form, one of these Latin square assays was therefore equivalent to over four of the earlier ones.

Effect of time in filling the plates. Working with assays of this type, the much increased accuracy revealed a bias in the potencies obtained, in that the 'unknowns', when they were actually known, came out lower than expected.

It is well known that if inoculated plates are kept in the refrigerator before use, the zones of inhibition are much larger than ordinarily, probably because the growth of the bacteria is checked, or some of the bacteria are killed, even though the plates are given ample time to warm up to room temperature before filling. The influence of the time the plates stood before filling was accordingly tested (time of standing before filling). Another possible source of error was the variation in the length of time the inoculated agar was held at a high temperature before pouring into the plates (age of agar). Eighteen plates were, therefore, poured in turn and numbered as they were poured. Plates 1, 2 and 3 were regarded as being 2 min. old; 4, 5 and 6 as 4 min.; and so on. The time of standing was also regarded from a similar standpoint. The total time for pouring was of the order of 10 min., which might have had an appreciable effect on an inoculated agar held at 60° for 10–20 min. beforehand, but which would be negligible, we presumed, compared with the 1–3 hr. which would elapse before the plates were filled. The key to the experiment, together with the results, is given in Table 5.

All the test solutions contained 10 units/ml. However, one was regarded as the standard and went on the plate first in the first series of nine plates and last in the second series of nine plates. The other three were regarded as separate samples and placed on the plates in the order shown by the prefixes A, B and C.

In the first nine experiments the concentrations of the second, third and fourth solutions are expressed in terms of the first as standard; in the last nine experiments the first, second and third are expressed in terms of the last as standard. (Two plates, 5 and 17, were upset and unfortunately could not be included.)

The results are very striking. Collectively, they indicate that the time which elapses between filling with one sample and the next is of vital importance to the assay result. In almost every case there is a uniform drop from sample to sample. (It should be noted that plate 18 was the fourth plate filled by operator C, i.e. it marked the point where she changed from 'standard first' to 'standard last' and having regard to the fact that the whole experiment had had to be repeated because of a misunderstanding, we are inclined to think that it was filled erroneously.) Table 6 gives an analysis of variance for the first nine plates. The values for the missing plate were inserted by a method based on

Table 5. *Effect of time of filling, and other factors, on potency estimations*

Plate	Operator	Approx. age of agar (min.)	Results in order of filling	Approx. time of standing before filling (min.)
1	*A*	2	10·00; $_{A}$9·60; $_{B}$9·47; $_{C}$9·00	10
2	*B*	2	10·00; $_{C}$9·06; $_{A}$8·68; $_{B}$8·01	30
3	*C*	2	10·00; $_{B}$9·25; $_{C}$8·96; $_{A}$8·64	20
4	*A*	4	10·00; $_{C}$9·13; $_{A}$8·76; $_{B}$8·46	20
5	*B*	4	(10·00; $_{A}$9·04; $_{B}$8·38; $_{C}$8·11)	10
6	*C*	4	10·00; $_{B}$10·21; $_{C}$9·20; $_{A}$8·53	30
7	*A*	6	10·00; $_{B}$9·30; $_{C}$9·42; $_{A}$9·51	30
8	*B*	6	10·00; $_{A}$8·91; $_{B}$8·22; $_{C}$8·93	20
9	*C*	6	10·00; $_{C}$9·47; $_{A}$9·31; $_{B}$8·14	10
10	*A*	8	$_{A}$10·68; $_{B}$10·82; $_{C}$10·33; 10·00	40
11	*B*	8	$_{C}$10·68; $_{A}$10·68; $_{B}$10·56; 10·00	60
12	*C*	8	$_{B}$11·88; $_{C}$10·40; $_{A}$10·77; 10·00	50
13	*A*	10	$_{C}$10·88; $_{A}$10·52; $_{B}$10·66; 10·00	50
14	*B*	10	$_{B}$10·52; $_{C}$10·66; $_{A}$10·36; 10·00	40
15	*C*	10	$_{A}$11·12; $_{B}$10·45; $_{C}$9·86; 10·00	60
16	*A*	12	$_{B}$12·06; $_{C}$11·93; $_{A}$11·12; 10·00	60
17	*B*	12	—	50
18	*C*	12	$_{C}$9·46; $_{A}$9·69; $_{B}$9·14; 10·00	40

The figures for plate 5 are the values inserted for the missing values.
The prefixes *A*, *B* and *C* relate to the three samples.

Table 6. *Analysis of variance of logarithms of results in Table 5*

Source of variance	Degrees of freedom	Sums of squares ($\times 10^6$)	Mean squares ($\times 10^6$)	*F*	*P*
Operators	2	4334·740	2167·370	4·56	0·05
Time of standing before filling	2	815·629	407·814	—	—
Age of agar	2	274·073	137·036	—	—
Order of filling:					
Linear term	1	4608·000	4608·000	9·69	0·01
Quadratic term	1	249·185	249·185	—	—
Samples	2	407·629	203·814	—	—
Residual	13	6181·262	475·482	—	—
Total	23	16870·518			

the principle of least squares. Having inserted these, the total degrees of freedom become three less than otherwise, and this decrease of three occurs in the residual.

The results have been analysed on the assumption that the second, third and fourth results on each of the first nine plates have been assayed in terms of the first sample placed on each plate.

The sample effect is non-existent, as also are the order of taking the plate and the age of agar at pouring. The order of filling has its linear component (expressed in terms of logarithms to base 10) highly significant, but its curvature non-significant. The operator effect is significant at the 5 % level. One particular operator had a particularly slow-acting filler and took much longer as a consequence in filling her plates. This serves to emphasize the effect of the time which elapses during filling on the assay result.

The residual variance averaged over this experiment and a similar one is approximately 480, corresponding to a standard deviation of about 22. The units in which the analysis has been conducted have been logarithms to base 10 × 1000 (this transformation being made for simplicity in the computation). Accordingly, a standard deviation in the real units is 0·022, and when converted to the 95 % fiducial limit on an expected figure of 100 the upper limit is about 111. This is very close to the estimate made for the Latin square when first used.

It was clear, therefore, that if this time effect in filling were eliminated a satisfactory assay would be obtained. The simplest method of elimination would have been to make a constant correction to the potency for the first unknown, a similar correction of twice the magnitude to the second unknown, and a similar correction of three times the magnitude to the third unknown. Unfortunately, examination of a few plates showed that the magnitude of such corrections would not be constant for all plates. Another method of elimination would have been to substitute the standard for the third unknown, the standard being on the plate twice, once at the beginning and once at the end. This would allow a determination of the magnitude of the correction for each plate, but would reduce the number of unknowns being assayed from three per plate to two per plate. The cost per assay would thus be increased by 50 %.

The use of the quasi-Latin square. Yates (1937) has given a design for carrying out an experiment of the form 2^5 (five factors all at two levels) in an 8 × 8 square, which involves the partial confounding of the higher order interactions. This design can be adapted to our purpose. We consider that we have one factor at two levels (the high and low dilutions) and one factor at eight levels. This factor at eight levels can be regarded as made up as

(1) standard preceding unknowns,
(2) unknown 1 succeeding the standard by one time interval,
(3) unknown 2 succeeding the standard by two time intervals,
(4) unknown 3 succeeding the standard by three time intervals,
(5) unknown 3 preceding the standard by three time intervals,
(6) unknown 2 preceding the standard by two time intervals,
(7) unknown 1 preceding the standard by one time interval,
(8) standard succeeding the unknowns.

This leads to an arrangement such as that in Table 7. Ideally this should be re-randomized every time it is used, but when doing 20–30 plates a day this is impracticable. The most satisfactory compromise is probably to construct about 20–30 arrangements, and have separate forms printed similar to Table 7 for each. Appropriate templates for transferring the results from the main table to the smaller one and for filling the plates would also be needed. The arrangement to be used in any particular assay can be selected with a table of random numbers. In Table 7, which has been used in all the assays of this type reported in this paper, the restriction has been imposed that in all rows and all columns the high and low levels alternate. This was to assist the operator in filling the plate, but a completely random arrangement is to be preferred on theoretical grounds. Randomization in practice can be carried out by drawing numbers from 1 to 8 from a hat, and rearranging the rows in the order thus given, and repeating this process for columns.

Table 7. *Form of entry of results from quasi-Latin square, and an example of its use*

Streptomycin assays

Assays: (1) (S_3)
(2) (S_1)
(3) (C)

Date............

Plate poured by..........................
Plate filled by ....
Dilutions made by.......................
Readings by...............................
Calculations by...........................
Checked by................................

Dilutions of samples: (1) 1/2·4 and (1) 1/24
(2) 1/36 (2) 1/360
(3) 1/40 (3) 1/400

Concentrations of standard: 10 and 1 units/ml.

1^F_L 10·9	2^I_H 14·4	S^I_L 11·1	S^F_H 14·9	3^I_L 10·9	3^F_H 14·6	2^F_L 11·1	1^I_H 15·2
2^I_H 14·2	1^F_L 10·5	3^F_H 14·0	3^I_L 10·7	S^F_H 14·5	S^I_L 11·2	1^I_H 14·6	2^F_L 11·1
S^F_L 10·5	3^I_H 14·2	1^I_L 10·2	1^F_H 13·7	2^I_L 10·6	2^F_H 14·5	3^F_L 10·7	S^I_H 15·1
1^I_H 13·5	2^F_L 10·3	S^F_H 13·7	S^I_L 10·6	3^F_H 13·9	3^I_L 10·5	2^I_H 14·5	1^F_L 10·9
2^F_L 10·7	1^I_H 13·5	3^I_L 10·0	3^F_H 13·6	S^I_L 10·6	S^F_H 14·0	1^F_L 10·4	2^I_H 14·4
3^I_H 13·2	S^F_L 10·0	2^F_H 13·5	2^I_L 10·1	1^F_H 13·5	1^I_L 10·1	S^I_H 14·2	3^F_L 10·7
3^F_L 10·0	S^I_H 13·6	2^I_L 10·2	2^F_H 13·6	1^I_L 10·2	1^F_H 13·9	S^F_L 10·6	3^I_H 14·1
S^I_H 13·6	3^F_L 10·0	1^F_H 13·5	1^I_L 10·2	2^F_H 13·7	2^I_L 10·5	3^I_H 14·0	S^F_L 10·8

S_L	S_H	1_L	1_H	2_L	2_H	3_L	3_H
11·1	14·9	10·9	15·2	11·1	14·4	10·9	14·6
11·2	14·5	10·5	14·6	11·1	14·2	10·7	14·0
10·5	15·1	10·2	13·7	10·6	14·5	10·7	14·2
10·6	13·7	10·9	13·5	10·3	14·5	10·5	13·9
10·6	14·0	10·4	13·5	10·7	14·4	10·0	13·6
10·0	14·2	10·1	13·5	10·1	13·5	10·7	13·2
10·6	13·6	10·2	13·9	10·2	13·6	10·0	14·1
10·8	13·6	10·2	13·5	10·5	13·7	10·0	14·0
85·4	113·6	83·4	111·4	84·6	112·8	83·5	111·6

$$1_H + 1_L - S_H - S_L = D = -4{\cdot}2$$
$$1_H - 1_L + S_H - S_L = B = 56{\cdot}2$$
$$\text{Antilog } D/B = 0{\cdot}8420$$

$$2_H + 2_L - S_H - S_L = D = -1{\cdot}6$$
$$2_H - 2_L + S_H - S_L = B = 56{\cdot}4$$
$$\text{Antilog } D/B = 0{\cdot}9368$$

$$3_H + 3_L - S_H - S_L = D = -3{\cdot}9$$
$$3_H - 3_L + S_H - S_L = B = 56{\cdot}3$$
$$\text{Antilog } D/B = 0{\cdot}8526$$

Titre of samples: (1) 20·3 units/ml.
(2) 337 „
(3) 341 „

Preferred method of assay of streptomycin

The following is the preferred method for dealing with about 60–90 assays (20–30 plates) per day. Sufficient nutrient agar (Bactopeptone, 5 g.; Difco beef extract, 3 g.; agar, 15 g.; distilled water to 1 l.; pH 8 before autoclaving at 15 lb. for 20 min.) for all the plates (140 ml./plate) is placed in a flat-bottomed Pyrex flask fitted with a ground-glass interchangeable neck, and the whole sterilized in the usual way. The flask contents are cooled in a water-bath at 60° for about 30–60 min. and then inoculated with a culture of *B. subtilis*. It has been found best to arrange that the inoculum is of approximately the same amount of the same age from day to day, and to this end agar slopes are prepared in 8 oz. culture bottles from 20 ml. of yeast-beef agar (above composition) which are sterilized at 15 lb. for 20 min. before sloping. Three such bottles are inoculated daily from a subculture carried in a Freudenreich bottle, which in turn has been prepared from the master culture of *B. subtilis* 288. Incubation of the slopes is at 32° for 5 days. The daily inoculum for the agar for the plates is obtained by adding 20 ml. of sterile distilled water to each of two or three of the 8 oz. slopes and shaking to suspend the spores. The mixed suspension from two bottles is usually sufficient to inoculate 3–4 l. of agar.

The inoculated agar is maintained at 60° for about 10–20 min. to afford some degree of pasteurization. About 140 ml. of the agar is then run on to the plate. The latter is previously warmed to 60° in an oven and removed as required for filling. The agar is poured mainly on to one part of the plate and run over the surface with as little tilting as possible; air bubbles are burst with a hot sterile needle. The plate covered with a glass sheet is laid on a perfectly flat table which is tested with a spirit level daily. The agar is usually completely set in 30 min. when disks are cut with a sterile no. 5 cork borer in positions determined by the plan marked on paper laid beneath the plate. The disks are removed with spear-shaped needles and placed in lysol.

The standard solution required for a week's work is made up to 100 units/ml. in pH 6·0 phosphate buffer, and each day suitable amounts are diluted in buffer pH 8·0 to give solutions containing 10 and 1 units/ml. Unknown solutions are similarly diluted in pH 8·0 buffer to give concentrations approximately 10 and 1 units/ml. (the ratio of concentrations of the two solutions must be 10:1).

The slopes of the log c-diameter curves for the standard and unknowns are sometimes appreciably different, and it has been confirmed that the pH's of such solutions often differ. The dilution of streptomycin sulphate solutions in pH 6·0 buffer with buffer of pH 8·0 gives solutions of pH about 7·6 or over. Many test solutions have pH values well above 8, and when diluted in pH 8·0 buffer the final value is about 8·2–8·4. For very precise work it would undoubtedly be desirable to adjust the pH of the standard and unknown solutions exactly to 8·0, but for routine research or production this would be too time-consuming.

Each operator uses her own cavity filler throughout. This consists of a 1–2 mm. capillary drawn out at each end and held by rubber tubing in a wider piece of glass tube, to the other end of which a rubber bulb is attached. The volume of the capillary should be about 0·05–0·06 ml. and experience will show the best shape for the jets. With a little care the capillary can be quickly filled and kept free from an air bubble, and by gently squeezing the bulb the contents of the capillary can be discharged into a cavity on the plate. Great importance is attached to ensuring that the same volume of liquid is placed in each cavity.

The operator has before her eight samples ('High' and 'Low' concentrations of each of three unknowns and the standard), and the plate lies on a special template based on Table 7 so arranged as to facilitate filling. The template is in fact Table 7 altered in scale to fit the actual plate. The rows (and columns) should be about 3·43 cm. apart, giving a total width and height of 24 cm. The terms 1^F_L, etc., are replaced by more vivid symbols. Thus four different colours can be used to represent the four different solutions, the early cavities and the late cavities can be represented by circles and triangles respectively, which can be made large and small to represent the high and low concentrations.

The filling order is as follows:

(1) The four cavities marked S^I_H, followed by the four cavities S^I_L. The suffixes H and L refer to the high and low concentrations and the superscript I means that these sets of four are the initial entries for the standard. These correspond to level (1) in the list of the eight levels given in the previous section.

(2) The four cavities marked 1^I_H, then the four cavities marked 1^I_L, corresponding to level (2).

(3) and (4) similarly for unknowns (2) and (3).

(5) The four cavities 3^F_L and then the four cavities 3^F_H, corresponding to level (5). The superscript F means that these sets are the final entries for this sample.

(6), (7) and (8) similarly for unknowns (2) and (1) and the standard, corresponding to levels (6), (7) and (8).

Between each solution the filler is washed with pH 8 buffer.

Filling is at first confusing, but with a little practice the operator soon acquires a steady rhythm, and after a few days filling becomes quite mechanical. It is essential that filling should proceed at a steady rate; a break of 2 or 3 min. makes the assay useless, whether in large-plate or Petri-dish work.

The plates are recovered and carefully placed in a horizontal position in a 32° incubator. Next morning the zone diameters are measured. After checking doubtful readings, the agar is peeled off the plate into a bucket of lysol and the plates and frames are immersed for an hour in this disinfectant. After washing thoroughly they are wiped with acid ethanol and dried in the oven 1–2 hr. at 60°. No attempt is made to ensure perfect sterility.

For transferring the zone diameter readings from the main table to the lower one in Table 7 eight templates, corresponding to the arrangement in question, are useful for laying on the main table to isolate the eight readings of S_L, etc.

Calculation of the results is greatly assisted by an adding machine. The necessary subsequent steps are carried out according to the schemes given at the lower right-hand side of the typical record sheet in which the results of an actual assay have been inserted (Table 7). The three sections in this record refer to the three unknowns. 1_H, 1_L, S_H and S_L, etc., refer to the totals of the corresponding columns to the left of the record and antilog D/B refers to the antilog to base 10 of the result obtained by dividing D by B. (Sometimes D/B is negative; for example, it might be $-0{\cdot}07473$. In this case one remembers that $-0{\cdot}07473$ equals $\bar{1}{\cdot}92527$ and finds the antilog of this, 0·8420.) The antilog D/B is the factor by which the concentration of the standards must be multiplied in order to give the concentrations of the unknowns (as placed in the cavities). Thus if the factor came out as 0·8420, the high and low concentrations of the unknown placed on the plate are 8·42 and 0·842 units/ml. Of course, these must be multiplied by the dilution factor in order to give the concentration of the original solution; i.e. the unknown solution may have been diluted with pH 8·0 buffer to dilutions of 1/2·4 and 1/24 in order to give concentrations approximately 10 and 1 units/ml. respectively. In this case the concentration of the original would be $24 \times 0{\cdot}8420 = 20{\cdot}2$ units/ml. If the method is used for penicillin assays, it is usual to work with the agar at pH 7·0, to use solutions at pH 7·0, to use concentrations of standard of 0·5 and 2·0 units/ml. and similarly for the unknowns. In this case, the ratio of the high and low concentrations being 4:1, it is necessary to find antilog 0·6021 D/B instead of antilog D/B to get the factor. In general, the factor is found from antilog $[(D/B) \times \log(\text{ratio of concentrations})]$ which for the streptomycin assays becomes antilog D/B.

A chart or set of tables can be constructed to give rapidly the value of the factor when D and B are known. Although at first confusing, the calculation, like filling the plate, soon becomes mechanical.

To illustrate the precision possible, Table 8 records the results obtained when three working standards S_1, S_2, S_3, and six solutions A to F were referred to a sample of pure streptomycin sulphate.

Table 8. *Reproducibility of assay potencies obtained by the preferred method*

Plate	Standard	S_1	S_2	S_3	A	B	C	D	E	F
1	827	334	125	—	223·5	—	—	—	—	—
2	827	334	118	—	—	673	—	—	—	—
3	827	341·5	122	—	—	—	345	—	—	—
4	827	342	123	—	—	—	—	512	—	—
6	827	355	122	—	—	—	—	—	—	828
7	827	—	121	20·7	216	—	—	—	—	—
8	827	—	124	20·4	—	670	—	—	—	—
9	827	—	122	—	—	—	331·5	—	—	—
10	827	—	121	20·7	—	—	—	491	—	—
11	827	—	121	20·6	—	—	—	—	81	—
12	827	—	120	—	—	—	—	—	—	846
13	827	340	—	20·8	218	—	—	—	—	—
14	827	350·5	—	—	—	680·5	—	—	—	—
15	827	337	—	20·2	—	—	341	—	—	—
16	827	334	—	20·7	—	—	—	511	—	—
17	827	333	—	20·0	—	—	—	—	78	—
18	827	344·5	—	19·6	—	—	—	—	—	898
	Means	340·5	122	20·4	219	674	339	505	79·5	857

Analyses of variance of three of the plates chosen at random are given in Table 9. The parallelism term is the interaction between treatments and levels, and has a total of seven degrees of freedom. Of these, however, one is partially confounded with columns, leaving six. In all cases the row and column mean squares are significant, frequently highly so, showing that the Latin square arrangement is removing from the error term a large part of the effect of the inhomogeneity of the plate. The average residual is 0·01749. It is interesting to note that the average residual obtained by ignoring the double restriction achieved by the Latin square, i.e. by pooling the sums of squares for rows, columns, and the residual, is 0·13973. The increase in accuracy through the use of the Latin square arrangement is given by the ratio of these residuals, viz. about 8·0 times.

Table 9. *Analyses of variance of three plates selected from Table* 8

Source of variance	Degrees of freedom	Assay no. 8		Assay no. 15		Assay no. 17	
		S.S.	M.S.	S.S.	M.S.	S.S.	M.S.
Rows	7	0·90610	0·12944	6·18859	0·88408	3·96000	0·56571
Columns	7	0·06110	0·00873	3·96859	0·56694	3·62000	0·51714
Treatments	7	1·19110	0·17016	0·81859	0·11694	1·84750	0·76393
Levels	1	215·72266	215·72266	197·75390	197·75390	244·14063	244·14063
Parallelism	6	0·13219	0·02203	0·37344	0·06224	0·15125	0·02521
Residual	35	0·26170	0·00748	0·83423	0·02385	0·74062	0·02116
Total	63	218·27485		209·93734		254·46000	

S.S. = sums of squares; M.S. = mean squares.

To calculate the internal error of an assay, we use the formula (cf. Finney, 1944)

$$s_m = \frac{2sd\sqrt{n}}{B^2}\sqrt{(B^2+D^2)},$$

where s_m is the standard error of the logarithm of the potency ratio, s is the standard error of an individual observation, d is the logarithm of the dilution ratio, n is the number of observations at each of the four points of the assay, and B and D have the meaning given in Table 7. In the case where the unknown has a potency close to that of the standard, D is small compared with B, and we get

$$s_m = \frac{2sd\sqrt{n}}{B}.$$

In the present case, an average value for s is $\sqrt{0{\cdot}01749}$, d is log 10 and therefore unity, n is 8, and B is about 60. The resulting value of s_m is 0·0124. For a large number of degrees of freedom we can take the 95 % fiducial limits as $\pm 2s_m$, or ±0·0248. These are the limits on the logarithm of the potency ratio, so the limits on the potency ratio are 94·5 and 105·9.

We can also obtain an estimate of the overall error from Table 8. For the S_1, S_2, S_3 observation the 95 % confidence limits for a single assay are estimated as 96 and 104 %. The close agreement between the internal and external estimates makes it evident that there are no unsuspected sources of error.

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A Technique for the Quantitative Estimation of Soil Micro-organisms

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SUMMARY: Soil micro-organisms have been counted by a new technique whose essential feature is the suspension of measured amounts of soil in a molten agar gel from which small drops are removed and allowed to solidify as thin films on a haemocytometer slide of known depth. The instantaneous gelation of the agar ensures the fixation of the soil constituents in their original distribution. The films are dried and stained in a solution of acetic-aniline blue and permanent preparations made by subsequent dehydration in ethanol and mounting in euparal. If the suspension is of known dilution, since films of a definite volume contain a known quantity of soil, differential counts of a measured area of film will yield a quantitative estimate of soil micro-organisms.

The distribution of bacteria per microscopic field was found to be complex. The frequencies of bacterial colonies and of pieces of fungal mycelium form a Poisson series; those of the number of bacteria per colony form a logarithmic series; and those of the total number of bacteria per field fall into a negative binomial distribution.

The method appears to be capable of modification by the use of selective nutrient media for determining the quality of the microflora and possibly the percentage viability of the organisms present.

Since interest was originally aroused in the quantitative and qualitative estimation of the soil microflora, various methods have been developed to this end. It was early recognized that dilution plate counts, while giving consistent estimates of some soil micro-organisms and therefore having some value for comparing different soil samples, fail to give the total numbers, since the various media employed are selective in their action. Moreover, though the method has one great advantage in that living organisms only are counted, doubt always exists as to whether colonies of bacteria develop from one or more organisms and fungal hyphae from fragments of mycelium or from spores. On these considerations attention was turned to microscopic techniques of direct examination of soil.

Stained smears of soil as used by several workers, notably Conn and Winogradsky, give little accurate information of actual numbers owing to the difficulty of estimating the exact quantity of soil examined. Winogradsky (1925) attempted to estimate this quantity by the increase in weight of the microscope slide on which the smear had been fixed, while Conn (1918) sought to obviate the difficulty by spreading 10 mm.^{3} of soil suspension of known dilution over a sq.cm. of a microscope slide. However, it is impossible by this means to ensure an even distribution of particles and organisms throughout such smears, so that the counts give an inaccurate estimate of the soil population.

A definite advance is seen in the method of Thornton & Gray (1934), in which a known quantity of soil is added to a counted suspension of indigo particles of a size and density approximately similar to those of bacteria. Small drops of this mixture are placed on a slide, dried, and then stained with erythrosin. During the drying of a drop of soil suspension, surface-tension forces will alter the distribution of contained particles, but it is assumed that indigo particles and organisms will be affected similarly. From the ratio of numbers of bacteria to indigo particles in a definite number of microscopic fields, it is possible to estimate the numbers of bacteria/g. of soil.

In applying this method, it was thought that a blue stain would give better background contrast than the red or violet acid dyes previously used. Agar films were made and stained by aniline blue lactophenol. This proved to be an excellent bacterial as well as fungal stain. However, this entailed the use of a contrasting 'ratio' particle of which many were prepared but none proved satisfactory for various reasons.

A homogeneous distribution of both organisms and soil particles could be obtained in a soil suspension in agar and in thin films subsequently prepared from it. The viscosity of the agar prevented soil flocculation, and its instantaneous gelation prevented the disturbance of the particles by surface-tension forces during drying; and a count of a known volume of soil suspension could be made when the films were prepared on a haemocytometer slide of known depth. Counts from such films gave evidence that a random distribution of organisms had been achieved and the need for adding a counted suspension of particles was thus obviated, since the number of organisms/g. of soil could now be derived directly from counts from the agar film of organisms alone.

During subsequent work with this aniline blue-lactophenol staining method it was found that the quality of the staining was variable, although the same procedure was followed on all occasions. Moreover, the stain gradually faded if the preparations were kept for several weeks. The impermanent nature of the lactophenol mounts was also unsatisfactory. Dehydrating the films in ethanol after staining and mounting in Canada balsam proved unsatisfactory, for differentiation was poor and balsam proved to have too low a refractive index for satisfactory resolution. The expedient of drying the film on to the slide was adopted after measurements had shown that the film did not shrink laterally when dried, and so the lateral distribution of particles and organisms remained unaltered, though the film had dried to a negligible thickness.

A solution of aniline blue in aqueous phenol and acetic acid was used by Maneval (1936) for staining fungal mycelium, using lactophenol as a mountant. The authors found that clear differentiation as well as permanent preparations of soil films could be obtained by the use of this stain with subsequent differentiation and washing in ethanol and finally mounting in euparal. The technique finally adopted was as follows.

Description of the method

The method involves counting the organisms in a number of microscopic fields selected at random and calculating the total volume represented by the microscopic field observed. The latter is estimated by multiplying the area of the microscopic fields by the depth of the haemocytometer slide on which the films are prepared. This gives the volume of soil suspension observed. Hence, if the initial dilution of soil in agar be known, the number of organisms per g. of soil can be readily calculated by multiplying by a conversion factor, which is much simplified if the initial quantity of soil be so adjusted as to give a simple fraction of 1 g. of soil in the total number of microscopic fields observed. For observation under oil immersion the authors found that 0·05 μg. per 20 fields ensured a satisfactory density of organisms and soil particles per field. If the volume of 20 fields of 0·1 mm. depth be x ml. for a particular eyepiece-objective combination, then 0·05 μg./x of soil must be added per ml. of agar. Hence the average number of bacteria per 20 fields $\times 2 \times 10^7$ gives the number/g. soil. (For the authors' microscope the appropriate wt. of soil was 2·58 g.)

The soil sample is first sifted through a 2 mm. sieve and the required quantity weighed out, placed in a small crucible with 5 ml. of sterile distilled water and thoroughly ground up with a glass rod. The resultant suspension is then poured off into a 100 ml. sterile flask. The sediment is washed in 5 ml. sterile distilled water and the suspended matter poured off into the same flask. With care only the heavier sand fraction then remains in the crucible, but the procedure may be repeated if necessary. The soil suspension is then made up to 50 ml. with 1·5 % agar, previously filtered hot through a No. 1 Whatman filter-paper, sterilized and kept at a temperature high enough to prevent gelation. After the flask has been shaken vigorously and left for 5 sec. to allow sedimentation of the heaviest sand grains it is ready for use and should be used almost immediately, otherwise certain thermophilic organisms multiply rapidly, giving an inaccurate picture of the numbers and kinds of organisms present. A sample is pipetted from immediately under the surface of the suspension on to the platform of a haemocytometer slide of 0·1 mm. depth, immediately covered by a cover-slip and allowed to solidify. With a slide of 0·02 mm. depth large mineral particles caused films of greater thickness to form and hence give variable counts. A slide 0·1 mm. deep with a lower dilution of soil is preferable. The slide is then immersed in sterile distilled water and the cover-slip removed. Surplus agar, which has solidified in the moat of the haemocytometer slide, may be removed by running a sharp scalpel round the central platform; or, if a circular haemocytometer is used, the thick agar rim may be efficiently removed by a cork-borer. By agitation of the slide in the water the film is gently floated on to an ordinary microscope slide and allowed to dry. It is essential to use distilled water for this purpose to prevent precipitation on drying of salts normally dissolved in tap water. The films should be dried slowly at room temperature, as attempts to speed up drying generally result in their splitting.

One type of haemocytometer slide has parallel transverse grooves, which isolate a central rectangle ground to a lower level. The authors found it more convenient to form a square in the centre of this rectangle by grinding two grooves at right angles across it (see Fig. 1).

The dried films are then immersed for 1 hr. in the following stain: phenol (5 % aqueous) 15 ml.; aniline blue W.S. (1 % aqueous) 1 ml.; glacial acetic acid 4 ml.; filtered about 1 hr. after preparation. Other sulphonated triphenyl rosaniline dyes were tested and in general proved satisfactory. Since a standard dyestuff seemed desirable, British Drug Houses Ltd. water-soluble aniline blue was adopted. The specificity of the rosaniline dyes for basiphilic protoplasm was demonstrated by similar staining results with acid fuchsin.

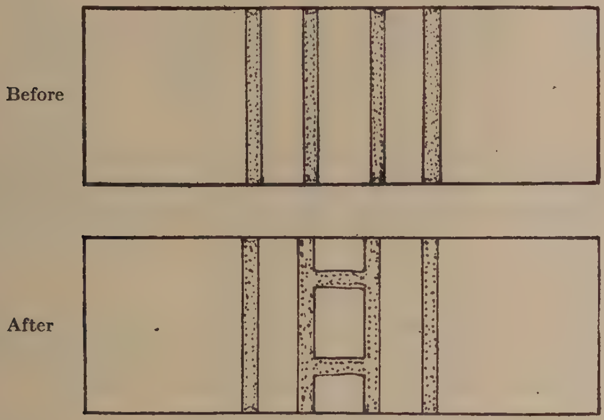

Fig. 1. Modification of haemocytometer.

The films are rapidly washed and dehydrated in 95 % ethanol, and permanent preparations made by mounting in euparal (Flatters and Garnett Ltd., Manchester).

For bacterial counts it is best to delimit the area of the microscope field by inserting in the eyepiece a glass disk ruled with a 2 mm. square.

Twenty random fields are then counted under oil immersion on each of four replicate slides, when multiplication by the appropriate conversion factor will give the number of organisms/g. of soil.

Verification of the method

It was thought desirable to test whether counts made by this method gave a true estimate of the total soil population. Accordingly, film counts were made of a soil sample previously sterilized and also of a replicate sample to which a known number of a particular species of bacterium was added from a counted suspension. The results in Table 1 show that the numbers of bacteria 'recovered' represent a very high percentage of those added.

The accuracy of this method depends on a uniform distribution of soil organisms throughout the suspension and subsequently in the agar film in order that this film may be a valid sample of the population under investigation. It depends also on the possibility of so standardizing the technique of preparation and counting that similar results may be obtained by different workers.

Table 1. *'Recovery' of counted suspensions of bacteria added to sterilized soil (millions/g. soil)*

No. added	No. in sterile soil + added counted suspension	No. in sterile soil alone	No. of bacteria counted	'Recovery' (%)
6440	6205	42	6163	95·7
1642	2760	1144	1616	98·4

Since there are many sources of variation inherent in this method, it is necessary to determine when variation between counts becomes significant, i.e. when the difference between counts may be assigned to differing soil populations rather than to experimental errors.

Errors may be ascribed to:

(1) heterogeneity of the agar-soil suspension;
(2) variation between aliquot fractions of the soil sample;
(3) variation between replicate films made from the same sample of soil;
(4) variation in observation between different observers; and
(5) indifferent staining and poor optical arrangements.

In order to investigate the differences in the microflora due to different manurial treatments and at the same time to provide data for the statistical analysis of these possible sources of error, counts were made of samples of soil from three of the 'classical' Barnfield plots. These have had consistent manurial treatment for the past 100 years. The plot numbers and treatment are 8–0, no manure; 1–0, farmyard manure; and 4–A, complete minerals. Samples from these three plots are designated A, B and C respectively. Sample C was divided into two parts C_1 and C_2. In Table 2 the count made on sample A is set out in detail showing the total number of bacteria observed in each of 20 microscopic fields. The total number of bacteria per field are analysed into their constituent colonies, the numbers per colony being shown.

Only the number of bacterial colonies and total number of bacteria per slide are given in Table 3 of the counts made on samples A, B, C_1 and C_2. The four counts made on replicate slides for each sample will be seen to be reasonably consistent.

From consideration of previous techniques of dilution counting ('Student', 1907), it was at first thought that the numbers of organisms observed per field should fall in a Poisson series. The required condition for a Poisson distribution is that the probability of an event occurring is exceedingly low, but the number of trials is so large that the total number of events occurring reaches an observable total. Suppose any microscope field to be divided into units of area comparable in size to a bacterium, there will then be an exceedingly large

Table 2. *Count of bacteria from sample of soil from Barnfield, plot A*

Slide		No. of microscope field																				Total no. of bacteria/slide
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	No. of bacteria within colonies	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	
		7	1	1	1	1	1	1	5	1	1	5	2	1	.	1	2	5	2	1	.	
		.	.	2	.	2	1	6	.	1	4	.	2	2	.	6	6	.	.	1	.	
		.	.	.	.	4	.	.	.	4	4	.	3	.	.	7	.	.	.	3	.	
		.	.	.	.	6	.	.	.	.	.	.	.	.	.	.	.	.	.	4	.	
	Total no. of bacteria/field	8	2	4	2	14	3	8	6	7	10	6	8	4	1	15	10	6	3	10	1	128
2	No. of bacteria within colonies	1	1	2	1	1	2	.	1	1	1	1	1	2	1	1	1	1	1	1	1	
		7	2	7	2	.	.	.	3	1	1	1	1	8	1	2	2	1	1	1	1	
		.	2	.	.	.	.	.	.	1	.	.	2	11	.	2	.	1	2	1	1	
		.	2	.	.	.	.	.	.	2	.	.	8	.	.	.	.	1	4	.	2	
		.	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	7	.	.	
	Total no. of bacteria/field	8	11	9	3	1	2	0	4	5	2	2	12	21	2	5	3	6	15	3	5	119
3	No. of bacteria within colonies	1	1	1	1	2	1	1	1	.	1	2	1	1	.	1	2	1	.	1	1	
		2	1	2	1	3	.	2	1	.	1	3	1	1	.	1	2	1	.	2	3	
		.	1	5	4	3	.	6	2	.	.	.	2	1	.	.	7	1	.	2	8	
		.	1	.	.	.	.	.	.	.	.	.	.	2	.	.	.	1	.	2	13	
		.	3	.	.	.	.	.	.	.	.	.	.	2	.	.	.	2	.	.	.	
		.	.	.	.	.	.	.	.	.	.	.	.	4	.	.	.	.	.	.	.	
	Total no. of bacteria/field	3	7	8	6	8	1	9	4	0	2	5	5	11	0	2	11	6	0	7	25	120
4	No. of bacteria within colonies	1	1	4	1	.	1	1	1	1	1	.	1	2	1	2	1	1	1	.	1	
		1	1	.	.	.	1	1	3	1	2	.	1	2	2	.	1	1	2	.	1	
		2	.	.	.	.	1	4	4	.	3	.	1	.	.	.	2	3	.	.	1	
		2	.	.	.	.	.	.	.	.	.	.	2	.	.	.	2	.	.	.	1	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	
	Total no. of bacteria/field	6	2	4	1	0	3	6	8	2	6	0	5	4	3	2	14	5	3	0	14	88

number of such areas. If the observation of each of these areas in turn represents a trial and the occurrence of a bacterium in any one of them an event, then the probability of such an event occurring is exceedingly low, since the total number of bacteria per field is small.

Table 3. *Count of bacteria in four samples of soil from Barnfield*

	No. of colonies/20 fields					χ^2 of colonies, $n=19$			
Slide	A	B	C_1	C_2		A	B	C_1	C_2
1	57	128	60	55		7·17	8·60	18·00	15·90
2	54	112	67	68		18·80	15·86	19·80	6·70
3	54	131	49	65		19·33	12·60	16·71	12·80
4	49	104	47	48		28·10	14·50	16·40	17·80
Av. no./slide	53·50	118·75	55·75	59	Total χ^2 on 4 slides $n=79$	72·55	56·68	81·12	55·52
Slide	Total no. of bacteria/20 fields					χ^2 of total no. of bacteria, $n=19$			
1	128	263	130	98		48·56	41·49	41·60	66·90
2	119	220	142	149		90·58	59·09	46·45	57·52
3	120	307	132	130		91·00	62·71	213·69	34·15
4	88	294	140	84		67·91	213·41	95·43	38·62
Av. no./slide	113·75	271	136	115·25					
No. of bacteria in millions/g.	2275	5420	2720	2305					

If this be true, the statistic χ^2, calculated as $\chi^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$ (where x = individual count of bacteria per field and $\bar{x}$ = mean value of each count and S implies summation), should be approximately equal to the number of degrees of freedom of the system (Fisher, 1946). From Table 3 it will be seen that the values of χ^2 thus obtained (the number of degrees of freedom, $n=19$) invalidate the assumption of a Poisson distribution.

However, when χ^2 was calculated on a 'recovery' count (see Table 4) agreement with the expectation of a Poisson series was obtained. In the latter case discrete organisms only were present, whereas in normal soils colonies of bacteria of various sizes occur, and it is clear that the distribution of bacteria in colonies provides a disturbing factor. There will thus obviously be a distribution within colonies to be taken into consideration on counts of normal soils. In order to assess the significance of the mean it becomes necessary to show that the distribution of colonies is Poisson, to determine further the type of distribution within colonies and hence to determine the distribution of total numbers of bacteria per field. Using the statistic χ^2 calculated as above, it was found that the distribution of colonies fitted a Poisson series. In Table 3 it will be seen that the values of χ^2 for counts on the individual slides on the whole approach the number of degrees of freedom, in this case 19. The values of χ^2 calculated on the slides grouped together with 79 degrees of freedom are also given and again do not deviate significantly from this value. In Table 7 the goodness of fit is demonstrated.

It then remained to determine the distribution within colonies. In discussion with Mr Quenouille it was suggested that these numbers might be distributed in the form of the logarithmic series investigated by Fisher, Corbet & Williams (1943) (see Statistical Note). In this the probability of colonies containing 1, 2, 3, ..., organisms are expressed by the successive terms of the logarithmic expansion for $-\log_\epsilon(1-x)$, i.e. $-\log_\epsilon(1-x)=x+\frac{x^2}{2}+\frac{x^3}{3}+\ldots+\frac{x^n}{n}+\ldots$. Since total probability must by definition be 1, the successive probabilities for 1, 2, 3, ... organisms will become αx, $\alpha\frac{x^2}{2}$, $\alpha\frac{x^3}{3}$ ... where $\alpha=\frac{1}{-\log_\epsilon(1-x)}$, and the relative frequencies may be obtained by multiplying each probability by the total number of colonies.

Table 4. '*Recovery*' *of counted suspension of bacteria added to sterilized soil*

Counted by two workers, M. and J.

Slide		No. of microscope field																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total	$\chi^2(n=19)$
1	M.	5	5	4	3	2	3	3	2	3	1	5	2	7	3	5	3	12	4	4	5	81	25·91
	J.	4	5	3	3	3	3	4	3	3	1	6	2	8	4	5	3	10	4	4	4	82	18·97
2	M.	6	4	3	5	2	11	6	6	12	5	5	7	4	6	7	5	6	3	8	2	113	22·40
	J.	5	3	3	6	3	12	5	5	10	3	7	8	2	6	6	5	6	2	6	3	106	31·73
3	M.	10	4	5	4	2	8	7	1	6	3	6	6	5	5	6	4	6	5	5	4	102	14·86
	J.	9	3	5	5	3	8	7	1	6	3	5	6	5	5	8	4	5	5	5	4	102	13·68
4	M.	3	5	6	6	4	3	3	9	6	9	5	6	5	4	2	6	1	2	3	5	93	18·61
	J.	1	5	4	7	4	5	2	9	5	9	5	5	7	3	3	6	3	3	4	6	96	17·75

In Table 8 a logarithmic series is fitted to the frequency distribution within colonies in each sample with Goodness of Fit tested by χ^2; and an excellent fit is obtained. That is, the distribution of colonies per field forms a Poisson series, and the distribution within colonies forms a logarithmic series.

The combined distribution, i.e. the distribution of total numbers of bacteria per slide (see Statistical Note) is proved to be in the form of a negative binomial expansion. Using this theoretical prediction the fit of this latter distribution to the total number of frequencies was tested by χ^2 (see Table 9) and again the hypothesis was verified.

Thus the total numbers per slide are seen to fit a negative binomial distribution; the parameters of which may be calculated, the standard error estimated, and the significance of the mean determined.

Thus from the counts of the three plots from Barnfield examined (Table 3) it appears that the microflora is more numerous in the plot treated with farmyard manure, whereas no significant difference may be observed between the plots to which minerals or nothing have been added. This has been borne out by several other unanalysed counts. Comparative counts by Thornton's ratio method gave slightly lower figures.

The method has also been used in an attempt to estimate the quantity of fungal mycelium in soil. The technique gives excellent staining of mycelium, but the estimation of its quantity is difficult owing to the small amount present and to the considerable variation in length of the fragments. In order to count

Table 5. *Count of pieces of mycelium in sample of soil from Barnfield plot A*

Slide		No. of microscope field																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total
1	No. of pieces of mycelium	1	1	3	2	.	.	.	.	1	2	.	1	1	1	.	.	.	.	.	1	14
	Total length in μ/field	25·6	25·6	512	115·2	.	.	.	.	25·6	70·4		25·6	57·6	76·8	.	.	.	.	.	51·2	985·6
2	No. of pieces of mycelium	2	.	.	.	.	.	1	.	1	.	1	1	.	1	.	1	.	.	.	.	8
	Total length in μ/field	128	.	.	.	.	.	25·6	.	51·2	.	115·2	64·0	.	32·0	.	76·8	.	.	.	.	416·0
3	No. of pieces of mycelium	.	.	.	1	1	1	2	2	.	1	1	.	.	.	.	2	.	.	1	.	12
	Total length in μ/field	.	.	.	102·4	19·2	25·6	281·6	172·8	.	44·8	204·8	.	.	.	.	422·4	.	.	96	.	1369·6
4	No. of pieces of mycelium	.	1	.	.	.	.	1	1	1	.	1	.	1	.	.	.	1	.	.	.	7
	Total length in μ/field	.	320	.	.	.	.	128	38·4	64·0	.	38·4	.	51·2	.	.	.	115·2	.	.	.	755·2

a sufficient number of fragments, it is necessary to examine the entire field of a low power objective, for which a new conversion factor must be calculated in order to express the quantity/g. Using a 2/3 objective and a ×8 eyepiece, only fungal mycelium is resolved, the finer actinomycete filaments being just below the limits of visibility. As for bacterial counts, 20 microscope fields on each of four replicate slides were counted. A measuring scale was inserted in the eyepiece, and the individual pieces measured, the quantity of mycelium being expressed as total length/g. soil.

Table 6. *Count of pieces of mycelium from three samples of soil from Barnfield*

	Total no. of pieces of mycelium per 20 fields				Total length (μ.) of mycelium per 20 fields			χ^2 of total no. of pieces of mycelium per 20 fields		
Slide	A	B	C_1		A	B	C_1			
1	14	12	8		985·60	947·20	614·40	20·29	28·00	12·00
2	8	15	6		416·00	1139·20	449·20	17·00	23·66	20·67
3	12	12	6		1369·60	1011·20	256·00	18·00	18·00	14·00
4	7	16	7		755·20	1152·00	358·40	13·00	21·50	13·00
Av. no./ slide	10·25	13·75	6·75	Av. length/ slide	881·60	1062·40	432·00			

The results of such an estimation made on the same slides as those used for the bacterial counts are set out in Tables 5 and 6. In Table 5 the detailed observations on sample A are given; in Table 6 only the total counts for each of the four slides of the three samples. The distribution of pieces of fungal mycelium like that of bacterial colonies fell into a Poisson series. The value of $\chi^2=\left[\frac{S(x-\bar{x})^2}{\bar{x}}\right]$, shows close approximation to the number of degrees of freedom (Table 6).

The nature of the soil microflora

The soil bacteria in films made according to this method are largely coccoid and adherent to the humic matter, few or none being attached to mineral particles (Pl. 1). These organisms may be in the form of large zoogl&oelig;al colonies (Pl. 1, fig. 1) or may consist of smaller clumps or single individuals (Pl. 1, figs. 2–7). Frequently groups of large cocci resembling Azotobacter are seen. Long rods have been but rarely observed in fresh soil. Staining varies in intensity, colonies generally being more deeply stained than discrete organisms, but this has not yet been correlated with viability.

The same variable staining is seen with fungal mycelium, where, on the other hand, there is strong evidence of correlation of intensity of staining with viability. Progressive loss of the protoplasm from the hyphae, due either to decomposition or to its migration to the hyphal tip, can be frequently observed (Pl. 1, figs. 8–10); indeed most of the hyphal fragments appear to lack organized contents. Such hyphae are stained purple in contrast with the deep blue coloration of those filled with protoplasm. This was confirmed by inoculating sterilized soil with fungal mycelium, allowing it to incubate for several days and making films from a sample of this soil. On these films only deeply stained fungal fragments were seen. On the whole, in normal soils mycelium is scanty and because of its filamentous nature and very variable length is not amenable to accurate statistics, though useful comparative results may be obtained. There were (Table 6) significantly fewer pieces of mycelium present on the plot with minerals than on the plots receiving farmyard manure and no manure.

Lengths of well-stained mycelium frequently have humic material adherent to their walls, probably through secreted mucilage (Pl. 2, fig. 4). This may have an important bearing on the formation of soil crumbs. Few fungal spores are seen. Fibres may be distinguished from hyphae by their lack of staining and their polarization colours under crossed nicols. Other plant tissue absorbs but little dye and at most has a greenish hue. Stained nematodes are sometimes seen and what are thought to be earthworm setae can be distinguished from fragments of mycelium by their tapering apices.

DISCUSSION

The counting method is presented as a new and more accurate technique for the estimation of quantity of bacteria and fungi, particularly for comparative analyses of different field soils. It is applicable not only to field samples but also to soil samples undergoing experimental treatment. It has, for example, been successfully used for observing the development of fungi in compacted blocks of soil mixed with resins, in the course of an investigation carried out for the Road Research Board D.S.I.R. (see Pl. 2, figs. 4–6). We also suggest the possibility of its wider application with slight modification. By using nutrient agar instead of plain agar for the soil dilutions and by incubating the soil films for several days in a damp chamber before drying and staining, excellent preparations of developing bacterial colonies (Pl. 2, figs. 1 and 3) and of actinomycete colonies, which stain beautifully, have been obtained (Pl. 2, fig. 2). By using various nutrient agar media in this way much information could be obtained about the specific nutritional groups of micro-organisms in the soil and of the relative frequency of dead and living organisms, inability to determine which is at present the chief disadvantage of direct counting methods as opposed to plate counts.

The direct method of examination and counting may well be applicable to solid environments other than soil, such as faeces, or sewage sludge. Moreover, soil films made in agar might be examined by a petrological microscope to identify the chief mineral elements in the soils and to estimate their relative abundance. A similar statistical analysis could be applied to this data, for a Poisson distribution of the rare minerals is to be expected.

This work was carried out during the course of an investigation on the decomposition of resins for the Road Research Laboratory by one author (P. C. T. J.), and in the course of an investigation for the Agricultural Research Council by the other (J. E. M.).

We wish to record our thanks to Dr H. G. Thornton, F.R.S., for his unfailing help and suggestions in the course of the work; to Prof. R. A. Fisher, F.R.S. for his advice in reading the manuscript; to Mr Victor Stansfield for photographs, and to Miss Mabel Dunkley for her assistance.

Statistical Note

By M. H. QUENOUILLE

Distribution of colonies per field—Poisson series

For a series of observations, the value of χ^2, calculated from $\dfrac{S(x-\bar{x})^2}{\bar{x}}$ can be used to test the hypothesis that the observations are distributed in a Poisson series. This statistic does not necessarily expose any systematic deviation from the Poisson series, but a comparison of the observed distribution of colonies per field with the theoretical distribution expected from a Poisson series shows (Table 7) no systematic deviation.

Table 7. *Poisson series fitted to colony counts*

No. of colonies	Sample A		Sample C_1		Sample C_2	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
0	7	5·5	0	4·9	4	4·2
1	8	14·7	20	13·7	9	12·4
2	24	19·7	24	19·1	16	18·2
3	20	17·6	12	17·8	23	17·9
4	11	11·8	9	12·4	17	13·2
5	8	6·3	8	6·9	8	7·8
6 and over	2	4·4	7	5·2	3	6·1
Totals	80	80·0	80	80·0	80	80·0
	$\chi^2_{(5)} = 9{\cdot}10$ $m = 2{\cdot}68 \pm 18$		$\chi^2_{(5)} = 12{\cdot}68$ $m = 2{\cdot}79 \pm 10$		$\chi^2_{(5)} = 5{\cdot}33$ $m = 2{\cdot}95 \pm 19$	

No. of colonies	Sample B		No. of colonies	Samples A and C	
	Obs.	Calc.		Obs.	Calc.
0–3	10	12·5	0	11	14·6
4	10	10·9	1	37	40·9
5	16	13·0	2	64	57·2
6	12	12·8	3	55	53·4
7	16	10·9	4	37	37·4
8	6	8·1	5	24	20·9
9 and over	10	11·8	6	12	15·6
Totals	80	80		240	240·0
	$\chi^2_{(5)} = 5{\cdot}18$ $m = 5{\cdot}94 \pm 27$			$\chi^2_{(5)} = 3{\cdot}41$ $m = 2{\cdot}80 \pm 11$	

Distribution of bacteria per colony—logarithmic series

On the following assumptions, which seem reasonably likely, the distribution of colonies consisting of 1, 2, 3, 4, … n individuals is given by the successive terms of the logarithmic series—$\log_e(1-x) = x + \frac{x^2}{2} + \frac{x^3}{3} + \frac{x^4}{4} + \ldots + \frac{x^n}{n} +$, with $x = \frac{y-pz}{qyz}$.

(i) In a fixed interval in time, the probability of a colony of size n becoming a colony of size $(n+1)$ is proportional to ny^n, i.e. is proportional to the size of the colony times a damping factor, which could be taken as unity.

Table 8. *Logarithmic series fitted to counts of bacteria per colony*

Bacteria per colony	Sample A		Sample C_1		Sample C_2	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
1	116	116·9	103	112·5	140	136·5
2	53	43·4	47	44·5	46	48·0
3	11	21·5	26	23·5	20	22·5
4	13	12·0	16	14·0	12	11·9
5	4	7·1	15	8·9	7	6·7
6	5	4·4	6	5·9	6	3·9
7 and over	12	8·7	10	13·7	5	6·5
Totals	214	214·0	223	223·0	236	236·0

Sample A: $\chi^2_{(5)} = 8{\cdot}81$, $x = 0{\cdot}743 \pm 0{\cdot}026$

Sample C_1: $\chi^2_{(5)} = 6{\cdot}54$, $x = 0{\cdot}794 \pm 0{\cdot}022$

Sample C_2: $\chi^2_{(5)} = 1{\cdot}94$, $x = 0{\cdot}704 \pm 0{\cdot}027$

Bacteria per colony	Sample B		Samples A and C	
	Obs.	Calc.	Obs.	Calc.
1	243	249·3	359	362·1
2	111	96·0	146	136·1
3	39	49·3	57	68·3
4	32	28·5	41	38·5
5	15	17·5	26	23·2
6	11	11·1	17	14·5
7 and over	24	23·3	27	30·3
Totals	475	475·0	673	673·0

Sample B: $\chi^2_{(5)} = 5{\cdot}46$, $x = 0{\cdot}770 \pm 0{\cdot}016$

Samples A and C: $\chi^2_{(5)} = 3{\cdot}91$, $x = 0{\cdot}752 \pm 0{\cdot}014$

(ii) In a fixed interval in time, the probability of a colony of size n breaking into two or more colonies is proportional to nz^n, i.e. is proportional to the size of the colony times an increasing factor, which could be taken as unity.

(iii) The probability that the break-up of a colony of size n will produce a colony of size m is proportional to

$$^{(n-2)}C_{(m-1)} \cdot p^{(m-1)} \cdot q^{(n-m-1)},$$

where $(p+q)=1$. This is the binomial probability that the division of n balls into at least two sets will result in a particular set having m balls in it.

(iv) The distribution is stable, i.e. it is not altering to any extent with time.

Comparison of the observed distribution with the theoretical (Table 8) shows no systematic deviation.

Distribution of bacteria per field—negative binomial series

If we assume both the Poisson and logarithmic series to represent the observed distributions very closely, then the expected distribution of bacteria per field may be shown to be successive coefficients of the negative binomial $(1-x)^{\beta}(1-xt)^{-\beta}$.

As yet there is insufficient data to test this distribution adequately. However, since no significant difference has been observed between samples A, C_1 and C_2 (see next section), these samples have been combined.

Table 9. *Negative binomial series fitted to bacterial counts*

	Samples A and C		
No. of bacteria	Obs.	Max. likelihood fit	Calc. fit
0	11	13·0	14·6
1	17	21·0	22·0
2	31	24·6	24·8
3	24	25·4	24·7
4	29	24·2	23·4
5	18	22·0	21·1
6	19	19·4	18·5
7	16	16·7	15·9
8	13	14·1	13·5
9	17	11·7	11·3
10	6	9·6	9·3
11	8	7·8	7·6
12 and over	31	30·5	33·3
Totals	240	240·0	240·0
		$\chi^2_{(10)}=9{\cdot}08$	$\chi^2_{(10)}=9{\cdot}66$
		$\beta=2{\cdot}2$	$\beta=2{\cdot}0$
		$x=0{\cdot}735$	$x=0{\cdot}752$

The negative binomial can be fitted by the method of maximum likelihood, or alternatively by using the observed values of m and x to calculate the parameter β. The fitted series for both methods are given in Table 9, where it is seen that both methods, in this case, give adequate fits to the observed figures.

Use of analysis of variance

(*a*) *On counts of colonies*

The analysis of variance can be used to test the technique used in counting colonies. A comparison of the variations between slides and the variations between counts on the same slide will indicate whether the variation between slides and the variation between replicate films made from the same sample of soil is likely to be large. We can also compare the samples C_1 and C_2 to investigate whether the variation between samples of the same soil is large.

Before carrying out the analysis of variance, the square roots of the counts were taken to normalize the data. Under this transformation, we will expect the mean square between counts on the same slide to have a value tending to 1/4, and any large deviation from this value can only be due to some fault in the technique. From the analysis of variance of the counts of colonies (Table 10) it is seen that the variation due to the technique is small, and furthermore that, although there is no significant difference between samples A and C, there is a large difference between sample B and the other samples.

Table 10. *Analysis of variance of colony counts*

	Degrees of freedom	Sum of squares	Mean square
Sample B *v.* other samples	1	39·816	—
Sample A *v.* samples C	1	0·479	—
Sample C_1 *v.* sample C_2	1	0·047	—
Between slides of the same sample	12	4·828	0·402
Between counts of the same slide	304	79·089	0·260
Totals	319	124·259	

(*b*) *On counts of bacteria*

The same technique can be used on the counts of bacteria. The mean square between counts on the same slide will, in this case, tend to the value $\frac{1}{4(1-x)}$, i.e. approximately 1. The analysis of variance is given in Table 11, the conclusions being the same as above.

Table 11. *Analysis of variance of bacterial counts*

	Degrees of freedom	Sum of squares	Mean square
Sample B *v.* other samples	1	95·622	—
Sample A *v.* samples C	1	1·832	—
Sample C_1 *v.* sample C_2	1	1·543	—
Between slides of the same sample	12	14·409	1·201
Between counts of the same slide	304	305·352	1·004
Totals	319	418·758	

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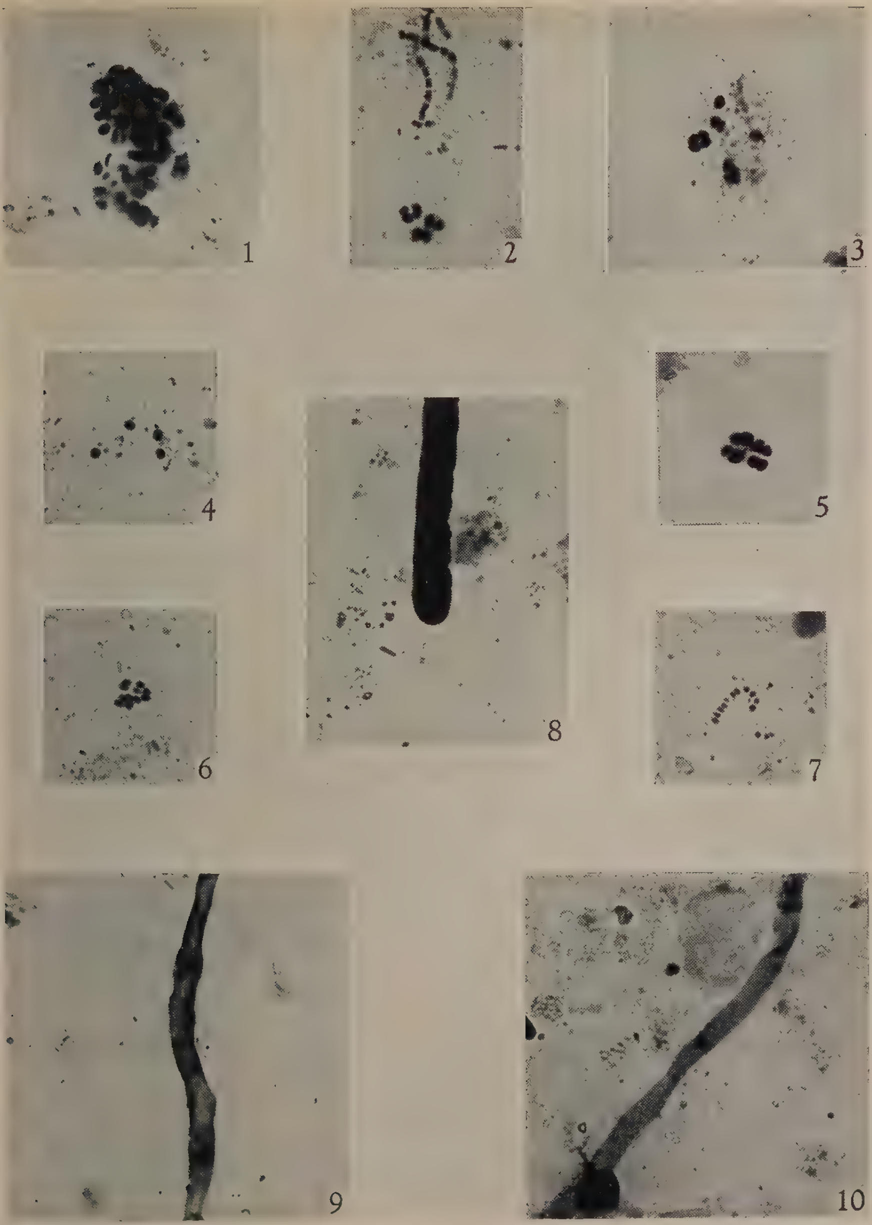
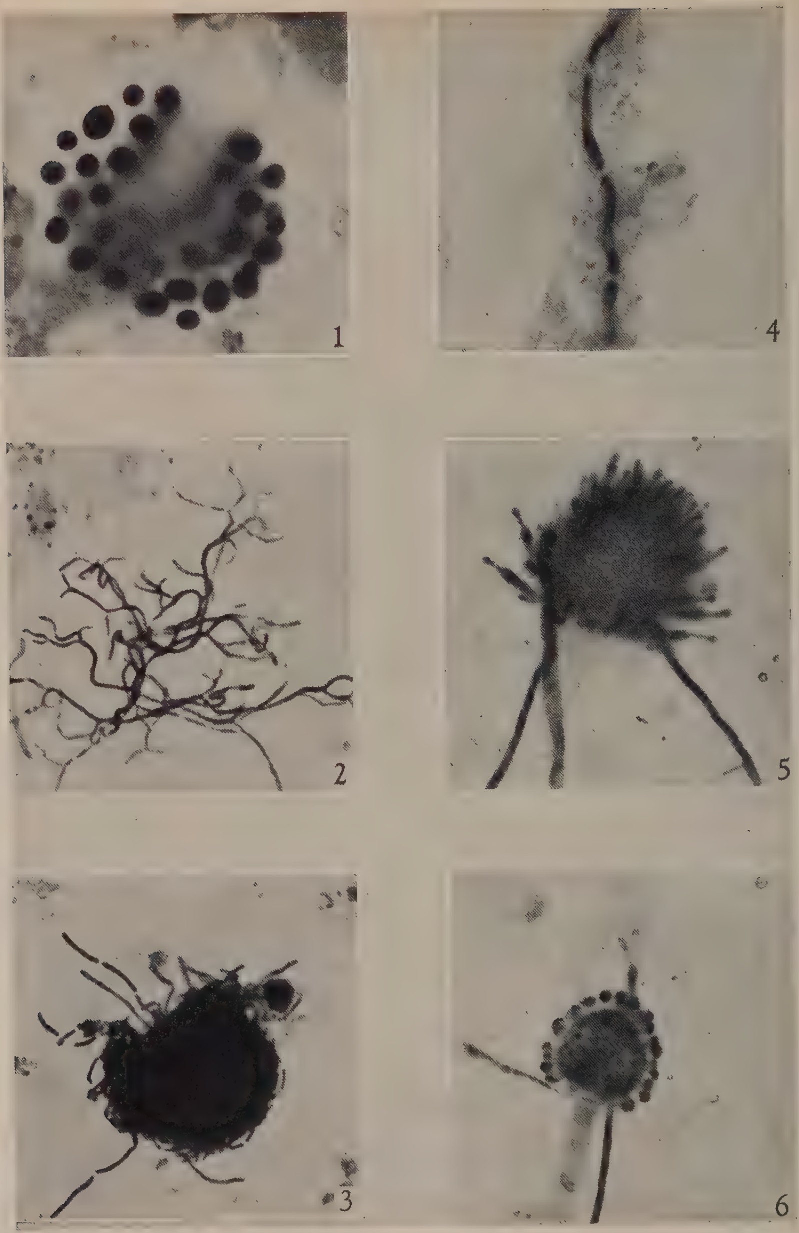


Figs. 1–10

P. C. T. JONES AND J. E. MOLLISON—A TECHNIQUE FOR THE QUANTITATIVE ESTIMATION OF SOIL MICRO-ORGANISMS. PLATE 1



Figs. 1–6

P. C. T. Jones and J. E. Mollison—A Technique for the quantitative estimation of soil micro-organisms. Plate 2

EXPLANATION OF PLATES

Plate 1

Figs. 1–7 from Barnfield manured plot. Figs. 8–10 from Broadbalk manured plot. Magnification × 1000.

Fig. 1. Large zooglœal colony.
Fig. 2. Small mucoid colony of cocci, and chains of rods.
Fig. 3. Colonies of cocci with adherent humic material.
Fig. 4. Single bacterial cells.
Fig. 5. Mucoid colony of large cocci.
Fig. 6. Colony of small cocci.
Fig. 7. Chain of cocci.
Fig. 8. Hyphal tip—Intense staining of preparation.
Fig. 9. Strand of mycelium with lysis or migration of protoplasmic contents.
Fig. 10. Hypha with only cell wall remaining (purplish staining of preparation).

Plate 2

Figs. 1–3. Nutrient agar film of allotment soil incubated 24 hr. Figs. 4–6. Film of crushed block of Harmondsworth brick-earth + 1 % Rosin. Magnification × 1000.

Fig. 1. Colony of large capsulated cocci with mineral crystal.
Fig. 2. Young actinomycete colony showing branching and fragmenting filaments.
Fig. 3. Young colony of rod-shaped bacteria.
Fig. 4. Fungus mycelium with adherent humus.
Fig. 5. *Penicillium* sp. conidiophore.
Fig. 6. *Aspergillus* sp. conidiophore.

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The Production of Spores by *Penicillium notatum*

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SUMMARY: *Penicillium notatum* gives high yields of spores on media containing carbohydrates, ammonia and an organic acid such as citric, tartaric, or succinic acid. The media are similar to those which are good for the production of penicillin, but the choice of organic acids is less restricted.

Of physical factors influencing the yield of spores the most interesting was variation in the volume of medium. Within certain limits the number of spores produced depended almost entirely on the volume of the medium and not on the surface area; i.e. the yield of spores/unit area depended on the depth of the medium. An increase in the concentration of metabolites above normal level did not greatly increase the yield of spores, and the addition of further nutrients to a previously used medium gave only a small second crop. The production of spores therefore appeared to be limited by the accumulation of toxic substances in the medium.

An important step in the production of penicillin is the preparation of the spore suspension of *Penicillium notatum* to be used as inoculum, and several groups of workers have already described media and conditions of growth which yield large numbers of spores (Moyer & Coghill, 1946; Gailey, Stefaniak, Olsen & Johnson, 1946). In the course of our work on penicillin it was desired to extend our knowledge of the factors governing the production of spores by *P. notatum*, in order to obtain the best results in large-scale work. This paper reports the results of investigations on methods for the growth and recovery of large numbers of spores, and on the effects on spore production of the composition of medium, temperature of incubation and the volume and surface area of the medium.

EXPERIMENTAL

Methods

Strain. The strain of *P. notatum* used was that used in these laboratories for the production of penicillin by surface culture. It was selected from the well-known Peoria strain, N.R.R.L. 1249 B21, and designated by us *P. notatum* M2.

Conditions of growth. To ensure reproducible results the spores must be grown under standardized conditions, removed completely from the medium and counted by a reliable method. In our experiments we secured the necessary conditions by a 'roll-culture' technique suggested to us by our colleague Dr A. Parker, followed by counting the spores in a haemocytometer. For this purpose 250 ml. quantities of medium were placed in 40 oz. milk bottles, 4% agar added and the bottles plugged and autoclaved. The bottles were allowed to cool to 40–50° and the agar set on the walls in an even layer by careful rotation under a stream of cold water. After testing for sterility by incubation for 3–4 days at 23–24°, the bottles were inoculated with 2 ml. of a suspension containing 5×10^7 spores/ml., carefully rotated to distribute the spores over the surface, and then incubated in an upright position in a constant-temperature room at 23–24°.

Counting of spore suspensions

(*a*) *Direct method.* After incubation of the cultures the spores were harvested by adding about 50 glass balls (*c.* 0·5 cm. in diameter) and 50 ml. water containing 0·1 % (v/v) Calsolene Oil H.S. (Imperial Chemical Industries Ltd., Dyestuffs Division) and then rotating the bottles mechanically at 100 r.p.m. until all the spores had been brought into suspension, as determined by inspection. After suitable dilution the spores were counted in a haemocytometer. Eighty small squares ($\frac{1}{400}$ mm.^{2}, $\frac{1}{10}$ mm. thick) were counted, and these usually included about 200 spores. An experiment was carried out to ascertain the numbers of bottles and counts that should be used to obtain repeatable results. In the experiment three bottles were treated in an identical fashion, four similar dilutions were made from each bottle, and four counts made on each dilution (Table 1).

Table 1. *Counting of spore suspensions from three bottles of medium*

(Medium: glycerol + molasses + peptone agar.)

The spores were suspended in 50 ml. of Calsolene oil-water and diluted 1/100; 80 $\frac{1}{400}$ sq.mm. haemocytometer squares were counted.

Bottle no.		Counts of dilution no.				Means per bottle
		1	2	3	4	
1		275	315	206	218	
		265	297	224	226	
		319	392	203	191	
		301	290	239	219	
	Means	290	323	228	213	261
2		325	248	337	354	
		324	271	318	298	
		362	270	281	203	
		245	212	295	211	
	Means	314	250	308	266	285
3		270	257	263	325	
		280	297	288	275	
		324	240	240	224	
		244	256	256	230	
	Means	279	262	262	263	267

There was no significant variation between bottles, but two samples out of one bottle gave significantly lower results than the others. The overall standard deviation between repeat counts from the same sample was 16 %, which is greater than the theoretical figure of 6 % for slide counting methods with mean counts of 250–300. It was concluded from the analysis of the data that to detect differences of 15, 30 and 100 % from a given average value 5, 2 and 1 counts respectively were required. However, owing to the possibility of an occasional abnormal sample or bottle, at least two bottles should be sampled in each case, and more than one sample from each bottle should be diluted and counted.

In each of the experiments described below, three or four bottles were inoculated, three samples were taken from the spore suspension prepared from one bottle; the samples were bulked, diluted, and two counts made on the resultant suspension. This procedure was repeated for the other bottles, so that the figures quoted are usually based on at least six counts. The yield of spores was calculated as the number/ml. of medium.

(*b*) *Densitometric method.* As an alternative and less laborious method of determining the numbers of spores in suspensions we tried a densitometric method, using a Spekker photometer. This method was suggested to us by our colleague Miss K. M. Wood, who had obtained encouraging results, using the M2 strain of *P. notatum* and the glycerol-molasses medium. We examined suspensions grown on the eight media referred to in Table 4. When the log of the concentration of spores was plotted against the log of the extinction coefficient straight lines were obtained which were parallel and lay very close together when two bottles of the same medium were taken. With different media, however, the pairs of lines were widely separated and were not parallel. These results show that although the method may be valuable for routine purposes it is unsuitable when a variety of media are being tested. The differences which we encountered could not be removed by using filters to compensate for difference in the colour of the spores or the liquid in which they were suspended.

RESULTS

Media containing glycerol, molasses, and peptone

The medium used was a glycerol molasses peptone medium based on that of Moyer & Coghill (1946), which preliminary tests had shown to be as good as any. The modification used by us had the following formula (figures show % (w/v) throughout): glycerol, 1; molasses, 1; peptone (bacteriological, Evans Medical Supplies, Ltd.), 0·5; NaCl, 0·5; KH_2PO_4, 0·006; $MgSO_4.7H_2O$, 0·05; $FeSO_4.7H_2O$, 0·0012; $MnSO_4.4H_2O$, 0·004; $CuSO_4.5H_2O$, 0·004. This medium was made up with tap water and adjusted to pH 6·5 with NaOH and solidified with 4 % agar.

Preliminary tests with 25 different media showed that this recipe gave the highest yield of spores. Some of these media contained various vegetable extracts only whilst others consisted of Czapek-Dox solution to which the extracts were added. The best yielded 20–80 million spores/ml. medium. The glycerol-molasses recipe usually yielded 300–500 million spores/ml. medium. Subsequently a number of synthetic media were tested, and some of these gave similar yields.

The optimal concentrations of salts and other constituents in glycerol molasses peptone media were tested by a simple factorial experiment in which media were made up containing the salts at 50, 100 and 200 % of the usual concentrations, and glycerol molasses and peptone in the same proportions (Table 2).

Table 2. *Production of spores with different concentrations of ingredients*

The yields are based on counts made on the 8th day. Counts made on the 3rd and 10th days gave similar results.

		Concentration of salts, % of normal		
		50	100	200
		Yield of spores (millions/ml. medium)		
Concentration of glycerol, molasses and peptone; % of normal	50	248	262	226
	100	218	384	326
	200	290	334	398

The best results were obtained with the two higher concentrations of ingredients. The difference between the yields given by these concentrations was too small to be detected in this experiment.

In another experiment two levels each of glycerol molasses peptone, volume of medium, and concentration of agar were used (Table 3).

Table 3. *Production of spores with different concentrations of nutrients and agar and volume of medium*

		Agar 3 %		Agar 5 %	
Vol. medium (ml.)		150	300	150	300
		Yield of spores (millions/ml. medium), 8th day			
Concentration of glycerol, molasses and peptone; % of normal	50	72	194	22	68
	100	198	340	100	265

The results show highest production of spores with normal concentration of nutrients and 300 ml. medium. The increase in the concentration of agar decreased the yield of spores. Counts made on the 4th day showed similar effects. In other experiments an increase in the concentration of the medium above the normal usually produced small increases in the yield of spores.

Synthetic media

Although the media so far described give high yields of spores a brief study of synthetic media was made, since they might be more reliable. Gailey *et al.* (1946) concluded that, of the substances in the glycerol molasses peptone medium, the peptone was the most important for the production of spores.

In an initial experiment spore production was measured on a basal medium containing the salts as used in the glycerol molasses peptone medium with the addition of sucrose (0·5 %), molasses (1 %), peptone (0·5 %), aqueous ammonia sp.gr. 0·880 (0·33 %), glycerol (0·5 %), acetic acid (0·75 %) (all % w/v). The combinations used, a half-replicate of the full factorial design, and the yields, are shown in Table 4. This type of experimental design is based on the work of Finney (1945). Although a half-replicate is not usually recommended for so few factors it was considered worth while in this case as the experiment was only exploratory, and by this means a representative set of treatments was obtained.

Table 4. *Production of spores on synthetic media*

The treatment combinations tested and the yields of spores are shown in the upper lines, a_0 indicating the presence of sucrose, a_1 the presence of molasses, b the presence of peptone, etc. In the analysis the combinations are shown diagrammatically, together with a summary of the mean effects produced by each main factor. 'Mean sucrose' represents the mean yields of all media containing sucrose, 'mean molasses' the mean of all with molasses, etc.

Combination of factors	a_1d_0	a_0bd_0	a_0cd_0	a_1bcd_0	a_0d_1	a_0bcd_1	a_1bd_1	a_1cd_1
Spores (millions/ml. medium) incubated 10 days	4	121	4	166	1	17	68	11

Analysis

		Yield of spores (millions/ml. medium)			
		d_0=glycerol		d_1=acetic acid	
		a_0=sucrose	a_1=molasses	a_0=sucrose	a_1=molasses
No ammonia	No peptone	—	4	1	—
	b=with peptone	121	—	—	68
c=with ammonia	No peptone	4	—	—	11
	b=with peptone	—	166	17	—

Main effects	Mean yields of spores (millions/ml. medium)
Mean sucrose (a_0)	36
Mean molasses (a_1)	62
Mean without peptone (–)	5
Mean with peptone (b)	93
Mean without ammonia (–)	49
Mean with ammonia (c)	49
Mean glycerol (d_0)	74
Mean acetic acid (d_1)	24

The largest effect was due to peptone, which markedly increased spore production. Glycerol was superior to acetic acid, which was the next factor in order of importance. No reliable conclusions could be drawn as to the comparison of sucrose and molasses and the effect of ammonia. The indications were that molasses might be superior to sucrose and that ammonia had no effect.

This experiment confirmed the importance of peptone as a factor for sporulation and also showed that glycerol was superior to acetic acid. In subsequent experiments glycerol was therefore added to all media.

Peptone is rich in amino-acids, which probably act as sources of nitrogen and carbon. It was thought that the peptone might be replaced by a suitable mixture of ammonia and a carboxylic acid. This possibility was suggested by the behaviour of synthetic media used for penicillin production, which were found to be greatly improved by the addition of 0·5–1·5 % (w/v) of citric acid. Media were therefore made up to contain 0·75 % (w/v) of various organic acids and the production of spores was measured. A parallel set of cultures was prepared with slightly different basal medium and the production of penicillin measured. The results of this experiment are shown in Table 5. The basal medium is seen to be not the most suitable for the production of penicillin and the titres recorded were relatively low. However, it is interesting that citric, succinic, tartaric and malic acids gave good yields of spores and penicillin while other acids gave many spores but no penicillin. There was in this series no instance in which there was a good yield of penicillin and a poor yield of spores. In the absence of an organic acid only mycelial growth occurred during the rather short incubation period.

Table 5. *Production of spores and penicillin on synthetic media*

The basal medium contained lactose 2 % (w/v), glycerol 1 % (v/v), NaCl 1 % (w/v), $(NH_4)_2SO_4$ 0·5 % (w/v), salt mixture 1 % (v/v), KH_2PO_4 0·1 % (w/v), NaF 0·001 % (w/v); with the addition of glucose 2 % (w/v) for sporulation or glucose 0·5 % (w/v) and phenylacetic acid 0·05 % (w/v) for penicillin production. All media were adjusted to pH 5·7–6·0 with KOH. The salt mixture contained $MgSO_4.7H_2O$, 50 g.; $FeSO_4.7H_2O$, 2 g.; $MnSO_4.4H_2O$, 1 g.; $ZnSO_4.5H_2O$, 1 g.; $CuSO_4.5H_2O$, 1 g.; $Co(NO_3)_2.6H_2O$, 2 g.; water, 1 l.

Acid added (0·75 %)	Spores (millions/ml. medium) (6 days)	Penicillin units/ml. (10 days)
Nil	0	0
Citric	246	53
Lactic	154	0
Succinic	282	42
Tartaric	316	58
Glycollic	178	Trace
Acetic	242	0
Oxalic	294	0
Malic	224	38

The results of other experiments which illustrate the high spore-yielding properties of synthetic media are illustrated by Table 6. Although the addition

Table 6. *Production of spores on synthetic media*

Basic medium same as for Table 4, incubated 6 days.

No.	Addition (%)	Spores, millions/ml. medium, after 6 days
1	Nil	4
2	Citric acid 0·5	140
3	Citric acid 1·0	440 362 (when repeated)
4	Citric acid 0·5, acetic acid 0·25	372
5	Glycerol 1·0, citric acid 0·5	592 280 (when repeated)
6	Glycerol 0·5, lactic acid 1·0	230
7	Glycerol 0·5. citric acid 1·0, $(NH_4)_2SO_4$ 0·25	258
8	$(NH_4)_2SO_4$ 0·25, lactic acid 0·5	6
Control	Glycerol + molasses + peptone agar	396

of more glycerol had an appreciable effect, the addition of citric acid gave the most striking results. The yields of spores seemed to be about equal to those obtained with the glycerol molasses peptone medium. These synthetic media contain a high concentration of carbohydrate and of carbon in the form of an organic acid; the presence of organic nitrogen seemed relatively unimportant. The best media for the production of spores were not unlike those which are suitable for the production of penicillin, but for the latter purpose the range of organic acids is more restricted.

The mechanism whereby the addition of organic acids increases the yield of spores remains unknown. Several authors have shown that the dry weight of mycelium produced by *Phycomyces blakesleeanus* is increased by addition of organic acids to the medium. Thus Leonian & Lilly (1940) concluded that the addition of organic acids facilitated the utilization of unfavourable nitrogen sources such as ammonium nitrate. Burkholder & McVeigh (1940), reporting similar results, suggested that these acids might be acting as a supplementary source of carbon. They also showed that the addition of organic acids influenced the type of sporangia which were produced.

Effect of different cultural conditions on spore production

The following experiments were carried out in the glycerol molasses peptone medium described above. Small-scale trials showed that spore production was best at 23–24°. The yields of spores were less at 20 or 28°, although at 28° growth and colour were apparently excellent. For example, after 6 days the spore yields were 320 and 80 million/ml. medium at 24 and 28°, respectively. The importance of making direct counts instead of judging by appearance was emphasized by this experiment.

Inoculation with 2 ml. of spore suspension, as described above, was found to be satisfactory. With 8 ml. of the same suspension spore production seemed rather less; this, however, was not studied in detail. The number of spores present was usually greatest after 4–5 days, cover by mycelium being attained after 1–2 days and a green colour appearing after 3 days. Table 7, which shows the results of an experiment on the effect of volume and depth of medium, illustrates this point. In this experiment the production of spores in milk bottles is compared with that in Winchester bottles.

Table 7. *Spore production with different volumes of medium in milk bottles* (M.B.) *and Winchester bottles* (Win.)

Volume of medium (ml.) ...	75		150		300		650		
Vessel ...	M.B.	Win.	M.B.	Win.	M.B.	Win.	M.B.	Win.	Mean
	Spores (millions/ml. medium)								
Days									
2	78	66	21	42	9	24	8	3	31
2	278	389	415	437	317	217	147	120	290
4	405	326	553	510	350	484	204	304	392
6	473	457	630	640	435	557	175	447	477
9	362	381	426	410	383	485	165	278	361

It will be noted that the counts were highest on the 4th and 6th days. The marked fall in number of spores by about the 8th day is characteristic. It may well be due to the germination of some of the spores, either *in situ* or after falling from the conidiophores, so that they are no longer recognizable as spores; a detailed examination of this point was not made.

Depth of medium

A preliminary experiment suggested that the production of spores per unit area of medium was roughly proportional to its depth. This effect was somewhat unexpected and does not appear to have been previously reported. A large experiment was therefore set up in order to investigate it further. The same volumes of medium (75, 150, 300 and 650 ml.) were used in milk bottles and 80 oz. Winchester bottles. The internal dimensions of the latter were approximately 11 cm. diameter by 23 cm. long. As the surface area of a Winchester is about double that of a milk bottle, comparable thicknesses were obtained in three instances. Counts were made on the 2nd, 3rd, 4th, 6th and 9th days, there being usually three replicate bottles (Table 7). The mean values for the 4th and 6th days, together with the yield of spores per unit area of medium surface, are given in Table 8.

Table 8. *Production of spores on different depths of medium*

(All spore counts are in millions.)

Volume (ml.)	Depth (mm.)	Surface area (cm.^{2})	Spores/bottle	Spores/ml. medium	Spores/cm.^{2} medium surface
			Milk bottles		
75	2	410	32,500	433	79
150	4	394	88,500	590	224
300	8	356	118,000	393	332
650	20	248	123,500	190	498
			Winchesters		
75	1	785	29,400	392	38
150	2	774	86,500	566	112
300	4	750	161,000	537	214
650	9	681	242,400	373	356

The yield of spores lay between 400 and 600 million/ml. of medium under the various conditions tested, except when 650 ml. medium were used, with a consequent diminution of the ratio of surface to volume. The production of spores per unit area appears to be fairly directly related to the depth of the medium up to depths of about 8 mm., when there is some falling off (Table 9).

Table 9. *Spore production with different depths of medium*

Depth of medium (mm.)	Spores/cm.^{2} (millions)		
	Winchesters	Milk bottles	Mean
1	38	—	38
2	112	79	96
4	214	224	219
8–9	356	332	344
20	—	498	498

The volume of medium is thus more important than surface area in governing spore production. A doubling of surface area (milk bottles to Winchesters) only increases the yield if the depth remains constant, and a similar increase in yield of spores would have been obtained if the larger volume of medium had been put in the milk bottles. With milk bottles, however, the surface area falls off with increasing depth, and the relationship was less marked when the thickness reached 20 mm.

It is interesting to note that the production of penicillin is also influenced by the depth of the medium. Thus with a medium containing corn-steep liquor and lactose in 250 ml. conical flasks the peak titres of penicillin were increased, and the yields of penicillin per cm.^{2} of surface with depths of 4, 8, 16, and 24 mm. were 45, 128, 243 and 250 units respectively, the optimum corresponding to a depth of about 20 mm. The shallower layers reach their highest titres much earlier than the deeper ones.

Factors limiting the production of spores of Penicillium notatum

The association between the depth of medium and number of spores produced per unit area suggested the presence of some factor related to the volume of the medium which controls the production of spores. Possible explanations are (i) that the larger volume of medium in the deeper layers would contain more nutrients and the yield should therefore be greater, (ii) the mould might produce substances which would accumulate in the medium and thereby prevent growth, and with larger quantities of medium more spores could be produced before the concentration became sufficient to interfere with the process.

Table 10. *Spore production on previously used media*

Bottles of single or double strength medium were inoculated and incubated 6 days. The spores were removed. In the unmelted set, fresh solutions of glycerol, etc., were added and the bottles rotated until the solution was absorbed. In the others the agar medium was melted, the mycelium removed and the medium reset on the walls.

			Spores, millions/ml. of	
			Single strength medium	Double strength medium
First crop		after 6 days	416	470
Second crop		after 4 days		
Unmelted	No addition		43	56
	With glycerol molasses		83	—
	With glycerol molasses and peptone		24	—
Melted	No addition		8	91
	With glycerol molasses		79	—
	With glycerol molasses and peptone		112	—

The experiments illustrated by Tables 2 and 3 suggest that merely increasing the quantity of nutrients above the normal would not be sufficient to account for the observed effect, for the effect of increasing the concentration of the nutrients usually was not as great as that obtained if the volume was doubled. Increasing the salts supplied, either directly or incidentally by increasing the quantity of agar, would be unlikely to account for the observed effect.

Both possibilities were examined by attempts to obtain a second crop of spores from once-used medium. It was found that if the first crop of spores was milled off, further production of spores was only slight; very small yields were also obtained when the medium was melted, the old mycelium removed, the medium resolidified on the walls and reinoculated. Rather larger yields of spores were obtained when more nutrients were added before resolidifying. The rather small size of the second crops appeared to be due to the presence of inhibiting substances. The pH of used media was found to be in the range of 6·0–6·5, which is similar to the original one. The addition of small quantities of acid or alkali did not increase the size of the second crop of spores. These statements are illustrated by a typical experiment recorded in Table 10. 'Melted' and 'unmelted' sets were included in order to find out whether the toxic substances preventing further sporulation were thermolabile. Our results are not conclusive on this point but are sufficient to indicate that heating had little or no effect upon them.

We wish to acknowledge the guidance we have received from Dr O. L. Davies in the planning and analysis of the results of some of the experiments described in this paper.

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An Asporogenous Variant of *Streptomyces griseus*

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SUMMARY: A non-sporulating form of *Streptomyces griseus* occurred spontaneously in culture. It appears to be a true variant of the species. Ultra-violet irradiation stimulated the variation. The variant was readily isolated on media containing relatively large amounts of organic nitrogen, but capable of propagation upon very simple media. The fact that the variant can be stabilized throws some doubt upon the validity of the present basis for classification of the actinomycetes.

It is now well known that *Streptomyces griseus*, in common with other actinomycetes, produces in culture media variants showing distinct cultural and biochemical features. Of these a grey and a white colony variant (see Pl. 1, figs. 1, 4) have been described (Schatz & Waksman, 1945). The former owes its colour to an abundant pigmented aerial mycelium, a feature which is associated with streptomycin production, whereas the white variant is less actively sporulating and produces insignificant amounts of streptomycin. A third colony type was also described by Schatz & Waksman which was non-sporulating and did not form streptomycin. This reverted spontaneously to the parent form.

As a result of examination of a large number of species in the *Actinomyces* group, Afanasiev (1937) found that in the pathogenic but not in the saprophytic types the amount of aerial mycelium produced was greatly influenced by the nitrogen content of the medium. Other workers (Waksman, 1919; Erikson, 1947) have under certain conditions obtained cultures of species of actinomycetes, which were devoid of any aerial mycelium, and this is usually considered to be a transitory condition, occurring particularly on media rich in organic nitrogen. However, sporadic appearances of an asporogenous form of *Str. griseus* in cultures of the purified grey variant grown on simple liquid media threw some doubt upon this conception. Its appearances could not be attributed either to an initial excess of organic nitrogen or to enrichment of the medium by autolytic products of the associated grey mycelium.

Isolation of the asporogenous form of Streptomyces griseus

Examination of a large number of strains of *Str. griseus* showed that the asporogenous form could be obtained and propagated on a variety of media. Its frequency was markedly increased by ultra-violet irradiation of *Str. griseus* spores. It represents a variation from the normal type as abrupt, discontinuous and complete as the white variant, and the cultures isolated are very stable. The same medium may give rise to normal grey, white and asporogenous growths, the last two appearing sometimes as sectors in grey colonies. It would therefore appear that the asporogenous form of *Str. griseus* may represent a mutation rather than a temporary response to a particular culture medium.

The asporogenous variants were purified by plating and, unlike those of Schatz & Waksman, were found to be very stable. Morphologically they consist of undifferentiated vegetative mycelium composed of hyphae of very uniform diameter, which tend in old cultures to break up into segments resembling oidia. Though they will grow on a wide variety of media the frequency of their appearance is governed to some extent both by the amount and the state of the nitrogen present. For example, freeze-dried cultures of the grey variant gave rise to permanently asporogenous colonies in the first generation on media containing peptone or glycine, but not on Czapek (sodium nitrate) agar or ammonium sulphate agar (Schatz & Waksman, 1945), and the proportion of the asporogenous type increased with increased amounts of peptone or glycine. The asporogenous variant comprised 12·6 % of the total colonies from a purified grey variant on a medium containing 1 % peptone and 0·5 % meat extract, but less than 1 % on a medium with 0·5 % glycine as the only source of nitrogen.

Though the asporogenous variant could not be isolated from grey cultures on Czapek or ammonium sulphate agar, it could be propagated upon these media, and was in fact able to grow, though poorly, on a synthetic medium containing washed agar and no added nitrogen. With peptone, glycine or ammonium sulphate as the nitrogen source the variant grew well. With from 0·5 to 1 % peptone (Pl. 1, fig. 2) growth was relatively rapid and profuse, and single colonies reached a diameter of 30 mm., when they lost their characteristic tenacious consistency and became friable, growing upwards in vertical ridges with marked pigment production; 1 % of glycine or ammonium sulphate produced a similar effect, but without pigment. With comparable amounts of sodium nitrate or with decreased amounts of glycine or ammonium sulphate, growth was much more scanty (Pl. 1, fig. 3).

Stability of the asporogenous variant

Reversion by purified cultures of the asporogenous variant does not occur readily, and was never found, during 5 months' observations, on media where growth was either abundant or very poor. Cultures have been kept for many weeks on peptone and glycine media, and on a nitrogen-free medium, without any detectable macroscopic or microscopic evidence of spore formation (Pl. 1, fig. 6). Partial reversion occurred most readily on media containing an inorganic nitrogen source in such concentration that growth was neither profuse nor too sparse. Thus, where the nitrogen content of the medium consisted of 0·005–0·05 % of ammonium sulphate or sodium nitrate a white powdery surface film developed on prolonged incubation, often appearing first as papillae or in concentric rings (Pl. 1, fig. 5). Subcultures from these colonies invariably reproduced the asporogenous form, with a varying proportion of feebly sporulating colonies resembling a poorly developed white variant. It was not possible to reproduce a parent grey variant on any medium. It is conceivable that even this feeble tendency to reversion might be eliminated by selection.

It therefore appears that the asporogenous growth of *Str. griseus* represents a true variant of the species, possessing greater stability than the parent form. The presence or absence of aerial mycelium has been used as a major criterion in classification of the actinomycetes, inability to produce this being a diagnostic feature of the *Actinomyces* group II*b* (Ørskov, 1923) and of the genus *Nocardia* (Waksman & Henrici, 1943). Consideration of the fundamental relationship between asporogenous and sporulating types and of the capacity of sporogenic forms to give rise to those permanently asporogenous might perhaps help to eliminate some of the present uncertainty in the nomenclature of this group of organisms, and place their systematic relationships upon a surer foundation.

The author wishes to express her thanks to Sir J. Drummond, F.R.S., and to Mr C. E. Coulthard for their helpful interest in this work; and to Mr P. H. Johnson for the photographs.

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EXPLANATION OF PLATE

Fig. 1. Grey variant of *Streptomyces griseus*; 10 days old; starch ammonium sulphate agar. ×1½.

Fig. 2. Asporogenous variant; 8 days old; peptone agar. ×5.

Fig. 3. Asporogenous variant; 8 days old; 0·5% glycine agar. ×5.

Fig. 4. Grey and white variants; 3 weeks old; starch ammonium sulphate agar. ×1½.

Fig. 5. Asporogenous variant; 6 weeks old; starch ammonium sulphate agar; natural size, showing scanty production of aerial mycelium in concentric rings.

Fig. 6. Asporogenous variant; 6 weeks old; 0·5% glycine agar. ×1½.

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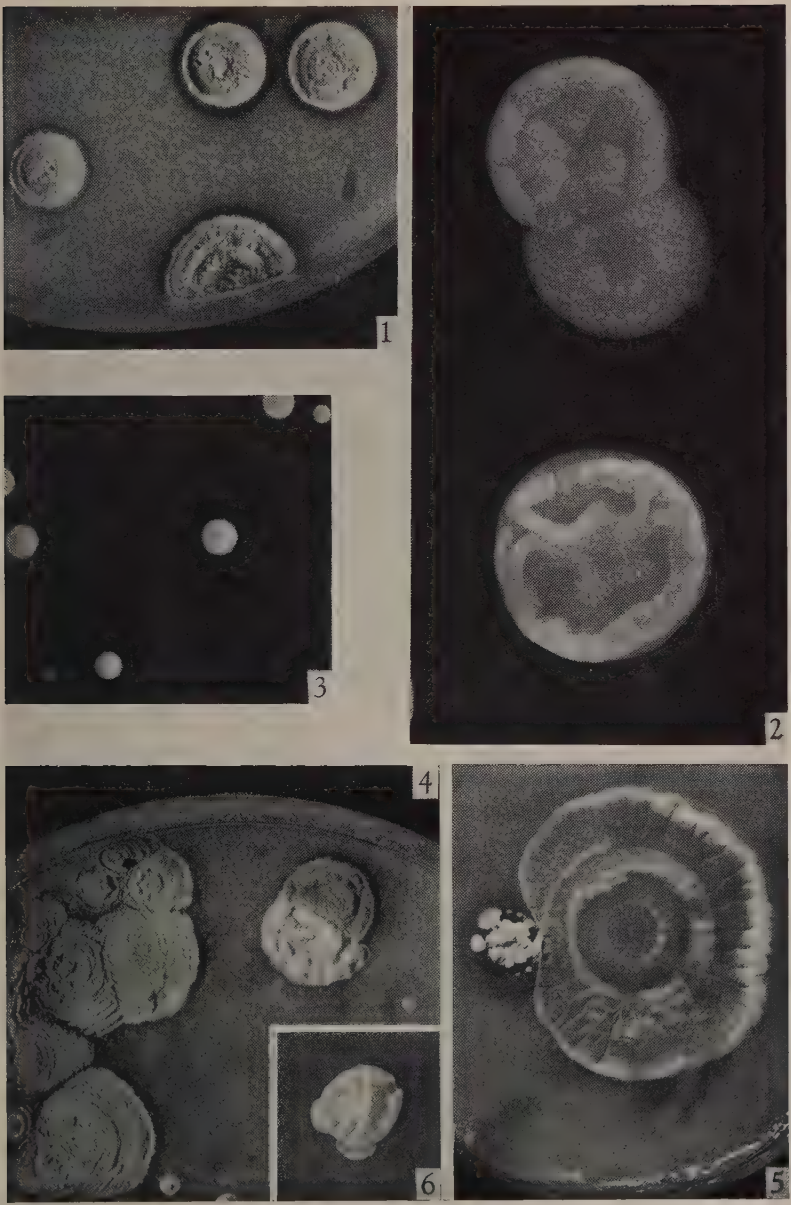

Figs. 1-6

J. C. Appleby—An asporogenous variant of *Streptomyces griseus*. Plate 1

The Cytology of Smooth and Rough Variation in Bacteria

By K. A. BISSET

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SUMMARY: In those members of the genera *Bacterium* and *Bacillus* studied, the constituent bacteria of Smooth cultures are typically unicellular, containing two chromatinic bodies. On division, a membranous septum is first formed, the bacillus subsequently dividing by constriction at this point.

The constituent bacteria of Rough cultures may comprise several cellular units, typically four, each containing a single chromatinic body and separated by membranous septa which, as growth proceeds, are transformed into true cell-wall septa, by the splitting of which the bacillus divides.

In previous papers (Bisset, 1938, 1939*a*, *b*) I described the structure of Rough and Smooth colonies, and the characteristics of the constituent bacteria. The structure of a Rough colony is that of the 'Medusa-head' colony of *Bacillus anthracis*, and the bacilli tend to be longer, and squarer at the ends, than do those of a Smooth colony, which is relatively structureless, and in which the bacilli lie separately. At cell division, transverse septa were observable in the unstained condition, only in Rough forms, the Smooth forms appeared to divide by constriction (Bisset, 1939*a*).

The present study is intended to extend these observations, and especially to discover, in these variants, the behaviour of the chromatinic bodies (Badian, 1933; Piekarski, 1938, 1939; Robinow, 1942, 1944, 1945).

METHODS

The strains employed were Gram-negative intestinal bacteria of various species including *Bacterium coli*, isolated during routine examinations of faeces. These were usually Smooth on isolation, and dissociation was induced in them by growth in meat broth for periods of a week or more, and subculture upon agar. Stock cultures of this type of organism were usually Rough. Gram-positive, sporing bacilli were isolated from dust in the laboratory, and were either Smooth or Rough upon isolation. Preparations were made from cultures aged 2–10 hr., grown aerobically upon nutrient agar at 37°.

At first, several methods of staining were tried, but the osmic acid fixation, acid-Giemsa staining, and Tannic-acid-violet techniques of Robinow (1945) were found to be best, especially for photography, and were eventually employed almost exclusively. The chromatinic bodies and cell membranes can, however, be demonstrated by many of the usual cytological staining techniques, especially by iron-alum haematoxylin. Water mounts were always employed for photography.

In the study of the Gram-negative bacteria of intestinal origin it was noticeable that the Smooth and Rough strains differed greatly in their staining reactions. Using the acid-Giemsa technique Smooth strains required only about 10 min. in N-HCl at 60°, and 0·75 hr. in the staining solution at 37°;

whereas Rough strains required from 0·5 to more than 1 hr. in the acid and then up to 5 hr. in the staining solution. Too short a period in the acid resulted in a uniform violet appearance when stained. The bodies were seen most clearly after about 3 hr. incubation for Smooth strains and about 5 hr. for Rough.

RESULTS

The observations which were made agree, for the most part, with those of Robinow (1945), but as the author's interpretation is somewhat different, a full description is given.

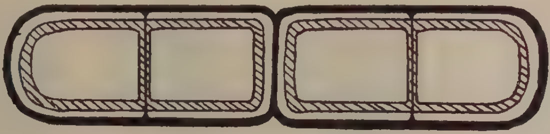
When stained by acid-Giemsa, the appearance of the two forms of the intestinal bacteria was quite distinct. The Smooth bacilli normally contained two chromatinic bodies, division of which usually preceded that of the cell, producing four bodies distinctly arranged in pairs. Between the two pairs of bodies, at the point of division of the bacillus, a septum, staining clearly with acid-Giemsa, was sometimes seen (Pl. 1, fig. 1*a*), and usually some degree of constriction was also visible at the point of division.

In the Rough forms there were usually four chromatinic bodies. The bacilli divided after the division of the bodies, and the point of division was marked, long before actual separation, by an unstained gap, which, as transpired subsequently, represented a septum, continuous with the cell wall and unstainable by Giemsa (Pl. 1, fig. 2*b*). The two halves of the bacillus were subdivided by septa which resembled those of the Smooth forms, and stained distinctly by Giemsa (Pl. 1, fig. 2*a*). Thus the Rough bacilli were, in fact, four-celled, and each cell was occupied by a single chromatinic body, sometimes in process of division.

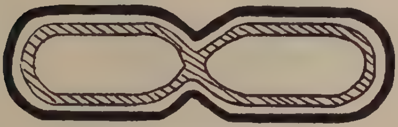
A large number of strains of Gram-positive sporing aerobes were also examined. The genus *Bacillus* is peculiar in that its members are naturally either of Rough or Smooth morphology. The author has never observed this type of variation to occur spontaneously in the group. Of the 40 strains examined, all the large-celled species were of Rough morphology, and produced 'Medusa-head' colonies, some of the small-celled species were Rough, and some Smooth.

The structure of the Rough intestinal bacteria was repeated with great clarity in these Rough Bacillaceae. The unit was a bacillus containing four chromatinic bodies, each divided from the next by a septum (Pl. 1, fig. 3). Division of the bacilli occurred at the middle septum, immediately after, or during the division of the four chromatinic bodies into eight. Bacillaceae of Smooth morphology also resembled Smooth intestinal bacteria, except that the chromatinic bodies were smaller in proportion to the size of the bacilli, which were also longer in proportion to their breadth.

When the various types of bacteria were stained with tannic-acid-violet to demonstrate the cell walls, an entirely different appearance resulted. In the Smooth forms, only the cell outline was stainable, sometimes showing constriction at the point of division (Pl. 1, fig. 4; Pl. 2, fig. 5). The septa, which stained clearly with acid-Giemsa, did not appear at all by this method, indicating that they were not of the same material as the cell wall, and were probably derived from the cell membrane. In the Rough forms stained by tannic-acid-violet it was obvious that the central division, an unstained gap in Giemsa preparations, was occupied by a septum continuous with the cell wall (Pl. 2, figs. 6, 7). The subsidiary septa, which stained with Giemsa, showed as faint shadows, presumably representing partial secretion of cell wall material within their thickness. No evidence either of centrifugal or centripetal 'iris diaphragm' formation of the new cell walls was observed. In these cells division was indicated by a slight indentation in the sides of the bacillus at the completed septum. There was no considerable degree of constriction.



1. Rough variant



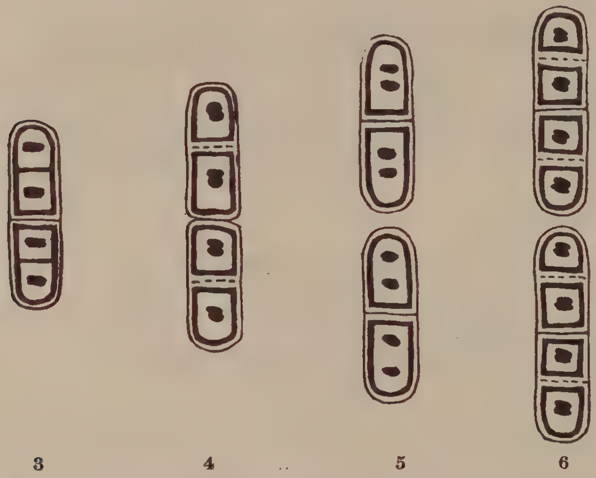
2. Smooth variant

Figs. 1 and 2. Membranes in dividing cells of Rough and Smooth variants. The cell wall is shown by heavy line, the cell membrane by hatched line.

DISCUSSION

The division of certain bacteria by septa staining well with basic dyes in their young stages, and of others by constriction, was recorded by Schaudinn (1902, 1903) and by many subsequent workers. The occurrence of multicellular bacteria is also fully established and has been well described by Robinow (1945) for some of the larger species. The process of cell division appears to occur in two main stages: first the formation of a transient septum, stainable with Giemsa, and presumably continuous with the cell membrane. The new cell wall forming the ends of the divided bacillus is then secreted by this membrane, but the manner in which this is accomplished differs in the two types of cell. In Rough bacilli the membranous septum is formed long before actual division occurs, two of the three partitions in a four-celled bacillus are of this nature (Figs. 1 and 3–6). The third, central partition is a true septum, continuous with the cell wall, and is secreted within the membranous septum. It is not stained by 'nuclear' dyes and the appearance of a fine, unstained line within the thickness of the strongly staining membranous septum is clearly shown in Schaudinn's illustrations. More recently it has been clearly described by Knaysi (1930). It is also visible in the unstained condition

(Bisset, 1939*a*). As the two new bacilli separate, a very slight degree of constriction accompanies the division of this septum. It has not as yet proved possible to determine whether the septum, as originally formed, is single or double, but its thickness does not appear to be greater than that of the cell wall. The claims of workers, from Schaudinn onwards, that transverse septa are formed either by a centripetal ingrowth or by a centrifugal outgrowth, are not supported by this work. The first appearance of the new cell walls was as shadows within the membranous septa, and occupying their entire area (Pl. 2, figs. 6, 7). This suggests that the former is composed of cell wall material secreted internally by the latter, exactly as transverse cell walls are reported to be formed in higher plants (Martens, 1937). In Smooth forms the membranous septum is formed immediately before the division of the unicellular bacillus, and instead of secreting an entire, new cell wall, in one piece, the existing cell wall grows inward, by constriction, to form the ends of the new cells (Figs. 2 and 7–10). Presumably this extension is secreted by the membranous septum, as is the cell wall by the cell membrane. This process also is clearly shown in Schaudinn's figures.

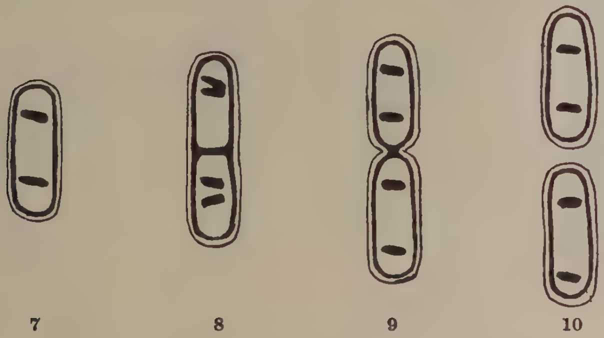


Figs. 3–6. Cell division in a Rough variant. The cell wall is shown by fine external line, the cell membrane by heavy line.

Robinow (1945) appears to have observed cell division by septation in a Rough *Bacillus* sp. and by constriction in a Smooth *Bacterium* sp., and to have assumed that these modes of division were respectively characteristic of the two groups of bacteria.

It has been suggested that the different degree of apparent constriction shown by the two types at the actual separation of the daughter bacilli, may be due to the more voluminous 'slime layer' of the Smooth forms, which forces apart the newly formed surfaces (Dr J. P. Duguid, personal communication).

Interpretation of the behaviour of the chromatinic bodies is less obvious. It is exceedingly difficult to judge the exact condition of a rapidly growing organism which can justifiably be considered to be the resting stage. In both types the bodies usually divide immediately before the bacillus, although exceptionally they may do so afterwards, and if the condition immediately after division is to be taken as the criterion of chromosome arrangement, then the Rough bacillus may be considered as possessing two cells, each with two chromosomes, exactly as in the unicellular Smooth bacillus. However, in any preparation of a Rough culture, the great majority of organisms appear to be in the four-celled condition, each of the original chromosome pairs being separated by a membranous septum and the entire bacillus halved by the new cell wall. At this stage the chromosomes proceed to divide, giving a total number of eight, before the division of the bacillus.



Figs. 7–10. Cell division in a Smooth variant. The cell wall is shown by fine external line, the cell membrane by heavy line.

Chromosome changes in bacteria have been observed before. The suggestion of chromosome reduction in connexion with sporulation has several times been made (Badian, 1933; Allen, Appleby & Wolf, 1939; Klieneberger-Nobel, 1945). The appearances recorded here, however, occur equally in sporing and non-sporing genera.

The parallel between colony structure of Smooth and Rough variants in the Bacteriaceae and that of the two morphological types in the Bacillaceae is shown to be based upon strong resemblances in minute structure. The significance of the variation, however, is still to seek.

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EXPLANATION OF PLATES

All × 3000.

Plate 1

Fig. 1. *Bact. coli*, Smooth, acid-Giemsa. Bacteria at various stages of division; membranous septum at *a*. Several bacteria are in the resting condition.

Fig. 2. *Bact. coli*, Rough, acid-Giemsa. Membranous septum at *a*; septum at *b* continuous with cell wall, showing unstained.

Fig. 3. *Bacillus* sp., Rough morphology, acid-Giemsa. Membranous and cell wall septa are clearly shown. Compare fig. 2.

Fig. 4. *Bact. coli*, Smooth, tannic-acid-violet, showing constriction of cell wall at point of division in numerous cases.

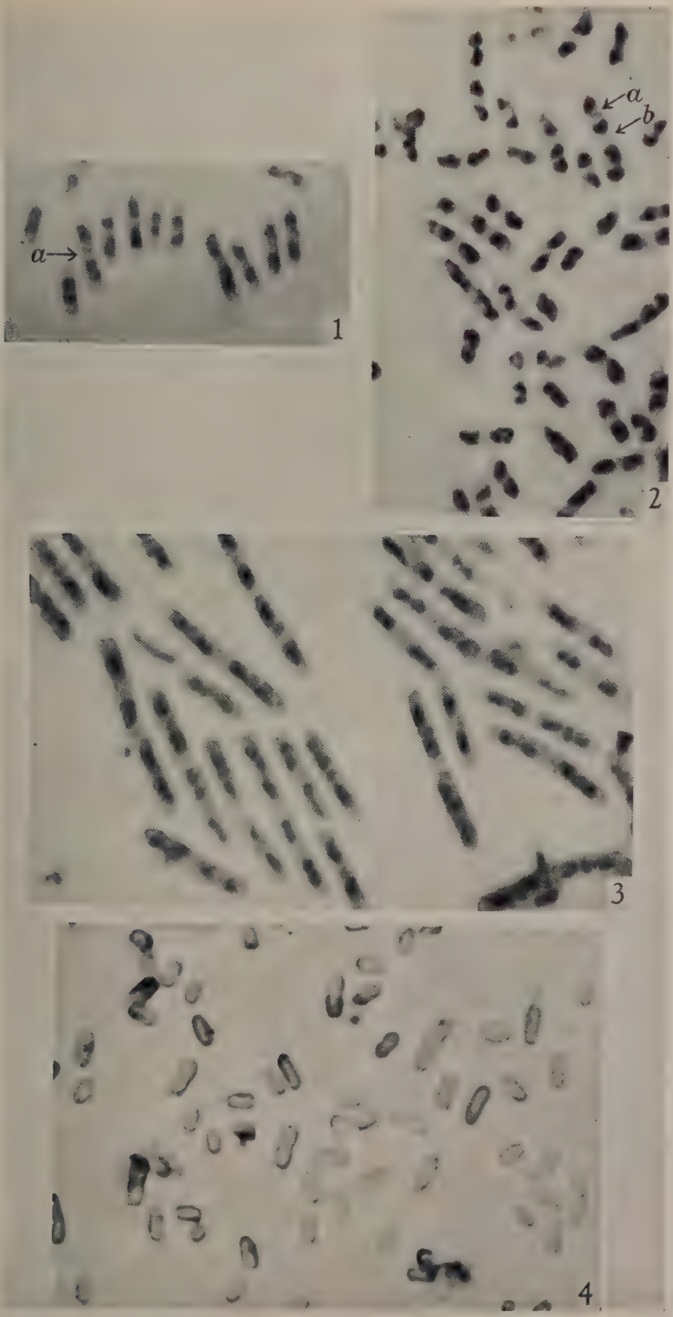
Plate 2

Fig. 5. *Bacillus* sp., Smooth morphology, tannic-acid-violet. Note the absence of transverse septa.

Fig. 6. *Bact. coli*, Rough, tannic-acid-violet. Note the transverse septa, and in right-hand bacillus, which is about to divide, the shadows of new septa forming. Compare fig. 2.

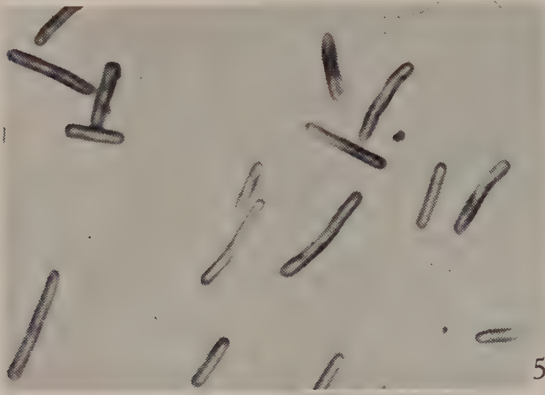
Fig. 7. *Bacillus* sp., Rough morphology, tannic-acid-violet. Note the transverse septa and the shadows of new septa forming in those about to divide. Compare fig. 3.

(*Received* 3 *September* 1947)



Figs. 1–4

K. A. Bisset—The cytology of smooth and rough variation in bacteria. Plate 1



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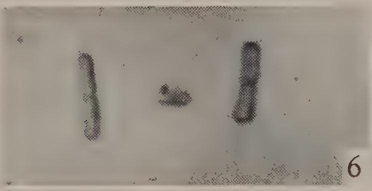

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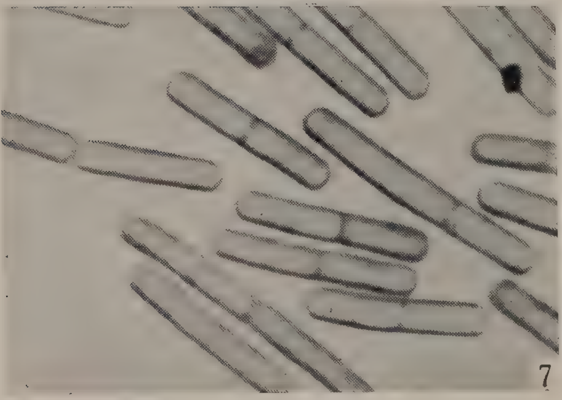

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Figs. 5–7

K. A. Bisset—The cytology of smooth and rough variation in bacteria. Plate 2

Studies on Giant Amoeboid Organisms

2. Nuclear Division and Cyst Formation in *Leptomyxa reticulata* Goodey with Remarks on the Systematic Position of the Organism

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SUMMARY: Excellent cytological preparations may be made from growth of *Leptomyxa reticulata* on cover-slips on a film of agar.

The organism is multinucleate. Each resting nucleus contains a deeply staining mass, the nucleolus, which is surrounded by a clear zone in which chromatin material is scattered. There is always a definite nuclear membrane. The nuclei divide simultaneously by intranuclear mitosis and the whole process is completed in a short time. At prophase the dispersed chromatin granules aggregate and later assume a thread-like structure and enter upon the metaphase. The formation of the spindle can be seen at the beginning of the metaphase. No centrosomes are seen at any stage. During anaphase the chromosomes begin to separate and it is difficult to count their numbers. The nuclear membrane disappears at late anaphase. At telophase the daughter chromosomes fuse together, the connecting threads break and the daughter nuclei are formed.

Under suitable cultural conditions and on certain strains of bacterial food supply, multinucleate cysts are produced in clusters. The process of cyst formation is described.

Leptomyxa reticulata Goodey, 1914 is widely distributed in soils of Great Britain (Singh, 1948). The study of the selection of bacterial food by *L. reticulata* has shown that under suitable cultural conditions and with certain bacterial associates these multinucleate organisms produce multinucleate cysts in clusters (Singh, 1948).

McLennan (1930) found *L. reticulata* associated with hop disease in Tasmania, but after a careful study concluded that it was a secondary invader and had no significance as a causative agent of the disease. These organisms were both in the root and on its surface, and in the lower part of the aerial bine. She thought that *L. reticulata* could penetrate the hop root either through the root hairs or by direct penetration through the epidermis, and after entering the root hairs, travel down into the cortical tissue and pass readily from one cell to the other.

For cultivating *L. reticulata*, McLennan (1930) used Erlenmeyer flasks with 100 ml. of tap water containing boiled wheat grains. In a synthetic food solution not containing carbon (Knop) and in soil infusion *L. reticulata* did not grow well. This she thought due to the insufficient development of bacterial food supply. It may be pointed out that nutrient media are often unsuitable either for isolation or growth of holozoic organisms because they may encourage the growth of inedible organisms and possibly of organisms producing toxic substances (Singh, 1945, 1946*a*, *b*, 1947*a*, *b*, *c*, 1948).

McLennan (1930, p. 36) says: 'When the organism found on the hop is compared with *Leptomyxa reticulata* the two forms are found essentially similar, and there is no doubt that the hop organism is correctly placed in this genus.' She concluded that the hop organism differed from *L. reticulata* Goodey in the following respects. (1) The cysts formed by the hop organism were much more irregular in shape and on the average much larger than those described by Goodey (1915). (2) The ectocyst in the hop organisms never approached the thickness attained in some cysts of *L. reticulata* figured by Goodey. On the basis of these differences she named the hop organism *L. reticulata* Goodey var. *humuli* (n.var.). The creation of this variety is probably not justified because the cultural conditions were such that mature cysts were unlikely to be formed. Judging from McLennan's diagrams, it seems certain that she was dealing with immature cysts or with those that were in the process of formation.

Goodey (1915) and McLennan (1930) have given a good general account of *L. reticulata*, although they could neither find the nuclear division in these multinucleate forms nor were they able to determine the mode of cyst formation. From their accounts it seems that one large individual gives rise to one multinucleate cyst.

The mode of nuclear division and cyst formation described in this paper may be of interest to systematists of the *Proteomyxa*, a group in which the great majority of forms have been very imperfectly studied.

MATERIAL AND METHODS

Leptomyxa reticulata was isolated from soil on non-nutrient agar (1·5 % agar in 0·5 % NaCl; pH 6·8–7·0) with *Aerobacter* strain 1912 (Singh, 1941, 1948). A pure line culture started from a single cyst was used throughout the work. *L. reticulata* forms cysts in large numbers only with certain bacterial associates.

L. reticulata did not grow very well on cover-slips covered with a suspension of a 2–4-day culture of a suitable bacterium in 0·8 % NaCl. In the presence of a thin layer of non-nutrient agar *L. reticulata* grew luxuriantly and produced cysts. Thin films of plain agar were made as follows. One or two drops of hot melted agar were put on a 2 × ⅞ in. cover-slip and spread into a thin film by quickly covering with a second slip. When the agar had solidified the upper cover-slip was gently removed, and one or two drops of a thick suspension of a suitable bacterium in 0·8 % NaCl were gently smeared on the agar surface. A small piece of agar containing *L. reticulata* was cut from an actively growing culture and put face downwards in the centre of the film. The cover-slips were kept in moist chambers in Petri dishes in order to prevent the drying of the agar and were incubated at 20–21°. Every precaution was taken to prevent contamination of the agar films. After 2–3 days, when some of the *L. reticulata* had moved on to the film, the piece of agar used for inoculation was removed.

Carnoy fixative was extensively used to make permanent preparations and gave better results than Bouin and Schaudinn. *L. reticulata* was fixed for 20–40 min. and then put into 95 % ethanol for 24 hr. The cover-slips were then brought through 70, 50 and 30 % ethanol to water, and at this stage the film of agar was gently removed from the cover-slip. As *L. reticulata* are large organisms, they sink into the agar and stick to the glass when the film of agar is removed after fixation. About 60–90 % of them remained on the cover-slips in a beautifully extended position as seen during life. The preparations were stained for *c.* 6 hr. in iron alum, overnight in haematoxylin and mounted in the usual way.

LEPTOMYXA RETICULATA

A general account of *L. reticulata*, its movements and division by plasmotomy has been given by Goodey (1915) and McLennan (1930). According to Goodey (1915) on nutrient-bouillon-agar *L. reticulata* has a waxy translucent appearance, the protoplasm being very compact and often disposed in an irregularly branched dendritic manner. When transplanted from agar into a liquid medium it assumes its normal shape and condition by stretching out into a thin sheet of almost transparent protoplasm. The abnormal shape observed by Goodey on nutrient agar was most probably due to the uncontrolled bacterial food which may have produced unfavourable metabolic substances.

When *L. reticulata* was grown on non-nutrient agar with a suitable bacterial food the organisms always stretched out into a very thin sheet of almost transparent protoplasm so that at times it was difficult to see them. Under suitable cultural conditions the size of *L. reticulata* was found to be much larger than those recorded by Goodey (1915) and McLennan (1930). When fully stretched an individual may attain a length of 3 mm. or more. As pointed out by Goodey, it assumes all kinds of fantastic shapes (Pl. 1, figs. 1–9), so that it may appear as though several species are present in the same culture. When the food supply is exhausted, and with drying of the agar, some of the individuals shrink so as to appear mycelial. In this form they remain alive for a month or more without forming cysts, and fresh cultures can be obtained from them without difficulty. The mycelial appearance was also sometimes observed in actively growing cultures, but under these conditions the protoplasm was nicely stretched out, and very branched. A single large *L. reticulata* looks like a small plasmodium of an endosporous Myxomycete, but without the regular reversible streaming movement which is found in the latter. The number of nuclei varies a great deal depending on the size of the individual; up to several hundred nuclei have been found in one large *L. reticulata*. In an actively growing culture a few very small individuals may be present (Pl. 1, figs. 4–8) containing 8–20 nuclei. They look like small true soil amoebae, and are the results of division by simple plasmotomy in which the daughter individuals produced are often of very unequal size (Pl. 1, fig. 10). As was noted by Goodey (1915) no nuclear division in the dividing individuals was observed either during plasmotomy or just afterwards. It is interesting to watch under a low-power microscope the process of division of the individuals by plasmotomy and the fusion of two or more of them into one, processes already recorded by Goodey.

Goodey (1915) says (p. 84): 'Stained examples show that the nucleus consists of a central deeply staining mass, the karyosome, surrounded by a clear zone which is often somewhat oval or spindle-shaped and does not seem to possess a definitely stainable membrane. Some sort of membrane is, however, present, for the nuclear sap is quite distinguishable from the surrounding cytoplasm. The process of nuclear division is quite obscure at present, but from the examination of a large number of living and stained specimens it seems that some kind of fragmentation of the karyosome takes place.' As noted below, the karyosome, which I call the nucleolus, does not seem to take any direct part in the intranuclear mitosis of *L. reticulata*. McLennan (1930) showed that each resting nucleus has a definite nuclear membrane, and the chromatic material is scattered evenly between the karyosome and the nuclear membrane. This observation of McLennan is in accord with my findings.

Nuclear division

Mitosis in endosporous Myxomycete plasmodium was so rarely observed by the earlier workers that they concluded that nuclei divide both by amitosis as well as by mitosis. With the development of better cultural methods, a few workers definitely proved that all the nuclei divide by mitosis simultaneously. The whole process is completed in a short time.

A large number of observations was made to find whether the nuclei in *L. reticulata* divide by mitosis, amitosis or both. At first no nuclear divisions were found. A few individuals were found containing nuclei very much smaller than are usually met with in individuals containing resting nuclei. It was thought that the nuclear division might either be completed within a short time or the nuclei might be dividing at some particular time of the day or at night. Accordingly, large numbers of cover-slip preparations were made at hourly intervals from 10 a.m. to 6 p.m. and from 8 to 12 p.m. Nuclear division was ultimately found in the material fixed during both the day and night. Out of the many thousands of large and small individuals examined only three large ones and one small one were found in the stage of nuclear division. All the nuclei in the small individual, numbering 20, were in the telophase of mitosis. All the stages of mitosis were found in each of the three large individuals containing several hundred nuclei. It seems that all the nuclei divide more or less at the same time and the process is completed in a very short time. Many *L. reticulata* were found in which the nuclei were only half the normal size. They apparently had just completed mitosis.

Details of mitosis

The resting nuclei in *L. reticulata* are either spherical or spindle-shaped (Pl. 2, figs. 20–22); and both kinds may be present in the same individual. The mitosis in some respects is like that found in vascular plants, the achromatic figure being devoid of centrosomes and asters. The nuclear membrane is a definite structure which can be seen up to anaphase (Pl. 2, figs. 23–32) and the nuclear division is intranuclear.

In each resting nucleus there is only one nucleolus. The first stage in the nuclear division is the aggregation of dispersed chromatin granules in prophase. The granules stain deeply with iron alum haematoxylin (Pl. 2, figs. 23, 24). The nucleus at this stage generally attains its greatest diameter. The deeply staining granules fuse together and begin to assume a thread-like structure. Soon afterwards, at metaphase, the chromosomes are compact and irregular and the spindle forms (Pl. 2, fig. 25). Later, the spindle is completely formed without any centrosomes (Pl. 2, figs. 26, 27).

During the anaphase the chromosomes begin to separate (Pl. 2, figs. 28–34). It is very difficult to count them accurately. In some nuclei it appeared that there were four chromosomes at each side of the spindle and in others in the same individual only two could be distinguished. In the late anaphase the nuclear membrane is not distinctly seen.

At telophase the spindle shrinks and appears as dark wrinkled lines (Pl. 2, figs. 35–38). The daughter chromosomes fuse together into a darkly staining mass. The connecting threads break and the daughter nuclei begin to form (Pl. 2, figs. 38, 39). The chromatin beings to fragment inside the nucleus, which at this stage again has a definite nuclear membrane. The nucleolus persists throughout anaphase and does not seem to take part in cell division. In telophase it either gradually fades away or is left out when the daughter nuclei begin to form. Sometimes more than one darkly staining mass can be seen inside each nucleus. The mode of formation of the new nucleolus could not be ascertained. It first appears as a small body which grows as the nucleus begin to grow.

The formation of cysts

Under suitable cultural conditions *L. reticulata* produces multinucleate cysts in clusters (Singh, 1948) resembling the sclerotium of an endosporous Myxomycete. The process of cyst formation is usually very slow, and mature cysts are produced only after several days. Parts of branching protoplasm are slowly drawn into dense and irregular masses which gradually become rounded (Pl. 1, figs. 12, 13). These rounded parts secrete an outer cyst membrane, or ectocyst, of varying thickness. The contents of the cyst continue to contract and secrete another wall, the endocyst, which is usually rounded. The ectocyst may be rounded or irregular (Pl. 1, figs. 15, 16). Usually the cysts break off from the parent individual when they are mature. Several cysts were seen in the process of formation attached to the parent individual in fixed preparations (Pl. 1, figs. 12, 13); these individuals showed no sign of nuclear division. The nuclei in the cyst-forming area were spherical and packed together, whereas the nuclei in the rest of the individual were either spindle-shaped or spherical and typical of resting nuclei. After the cysts are broken the parent individual continues a trophic existence. Sometimes rounded or irregular bits of protoplasm are cut off from a large individual, and give rise to double-walled cysts. As shown in Pl. 1, figs. 17 and 18, one ectocyst may contain up to ten endocysts, each of which is multinucleate (Pl. 1, fig. 19). Under good cultural conditions a single large multinucleate organism is completely broken into a number of cysts, the number depending on the size of the individual (Pl. 1, fig. 14). Up to 40 or more cysts may be produced from a single individual, which then resembles a sporangium. This formation of multinucleate cysts in clusters is a very interesting feature in the life cycle of *L. reticulata*. When the cysts are fully mature they can be easily separated from each other.

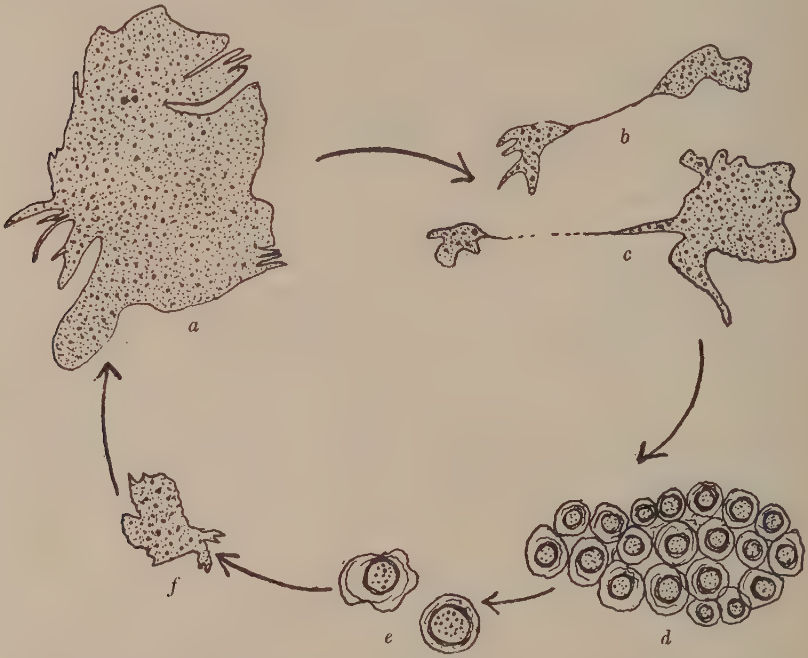

Fig. 1. Diagrammatic representation of the life cycle of *Leptomyxa reticulata*; *a*=fully grown multinucleate individual; *b* and *c*=division by simple plasmotomy; *d*=a cluster of multinucleate cysts formed from one individual; *e*=two multinucleate cysts; *f*=a small multinucleate individual after emerging out of a multinucleate cyst.

Excystation

When cysts are transferred to non-nutrient agar or to a few drops of 0·8 % NaCl on cover-slips supplied with suitable bacterial food they excyst readily. The process of excystation has been described by Goodey (1915). Multinucleate individuals have always been found to grow out of the cysts. Many of the cysts formed under good cultural conditions do not excyst and are probably dead.

THE SYSTEMATIC POSITION OF *LEPTOMYXA RETICULATA*

A number of mostly imperfectly studied forms which did not seem to fit in with the rest of the Rhizopods have been put into the group *Proteomyxa*. Since the discovery that *Leptomyxa reticulata* produces multinucleate cysts in clusters resembling the sclerotium in endosporous Myxomycetes, a considerable time has been spent in finding whether a flagellate stage occurs in its life cycle. As observed by Goodey (1915), a single cyst gives rise to one multinucleate individual which feeds on bacteria and grows into a giant form. The fusion of two or more individuals may result in the formation of one larger individual. The life cycle of *L. reticulata* (Fig. 1) seems to justify its inclusion in Rhizopoda.

It is curious that such a remarkable form as *L. reticulata* has been ignored by the writers of text-books on Protozoology. The mode of cyst formation and nuclear division in *L. reticulata* surely justifies the creation of a new genus as suggested by Goodey (1915). Goodey (1915), Sandon (1927) and McLennan (1930) have put this organism in the order Amoebaea. Schaeffer (1926) excludes rhizopods with reticulate pseudopodia from this order. In the present state of our knowledge, it is my opinion that the genus *Leptomyxa* should be put in the family Vampyrellidae (Doflein) belonging to the order Proteomyxa (Lankester), and ranking with the few well-recognized genera like *Arachnula* and *Vampyrella*, etc., thus bringing it nearer to the Amoebaea.

Leptomyxa flabellata (Goodey, 1915) has been isolated from a number of arable soils in Great Britain, but I have not yet studied the life cycle of this interesting form.

The observations of McLennan (1930) on the occurrence of *L. reticulata* in hop tissues is very interesting. It is possible that some of the Plasmodiophorales may have evolved from free living forms like *L. reticulata*.

This work was made possible by a grant from the Agricultural Research Council to whom the author's thanks are due. It is a pleasure to express my sincere thanks to Dr H. G. Thornton, F.R.S., and Miss L. M. Crump for their interest in this work.

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EXPLANATION OF PLATES

Plate 1

Leptomyxa reticulata. Figs. 1–18 are drawn from the same magnification. The magnification of fig. 19 is indicated below it. Cover-slip preparations fixed in Carnoy and stained with iron alum haematoxylin.

Figs. 1–9. Individuals of different sizes and shapes.
Figs. 10, 11. Division by simple plasmotomy.
Figs. 12–14. The formation of multinucleate cysts.
Figs. 15, 16. Typical double-walled mature cysts.
Figs. 17, 18. Cysts containing several endocysts.
Fig. 19. A part of the endocyst highly magnified to show the nuclei.

Plate 2

Intranuclear mitosis in *Leptomyxa reticulata*. Cover-slip preparations fixed in Carnoy and stained in iron alum haematoxylin.

Figs. 20–22. Typical resting nuclei.
Figs. 23, 24. Prophase stages in mitosis showing the aggregation of chromatin in one place.
Figs. 25–27. Metaphase stages and the formation of spindle.
Figs. 28–34. Anaphase stages and the separation of chromosome to two poles of the spindle. The nuclear membrane has disappeared in the late stages.
Figs. 35–38. Telophase stages and the formation of two daughter nuclei.
Fig. 39. Four small nuclei just after mitosis.

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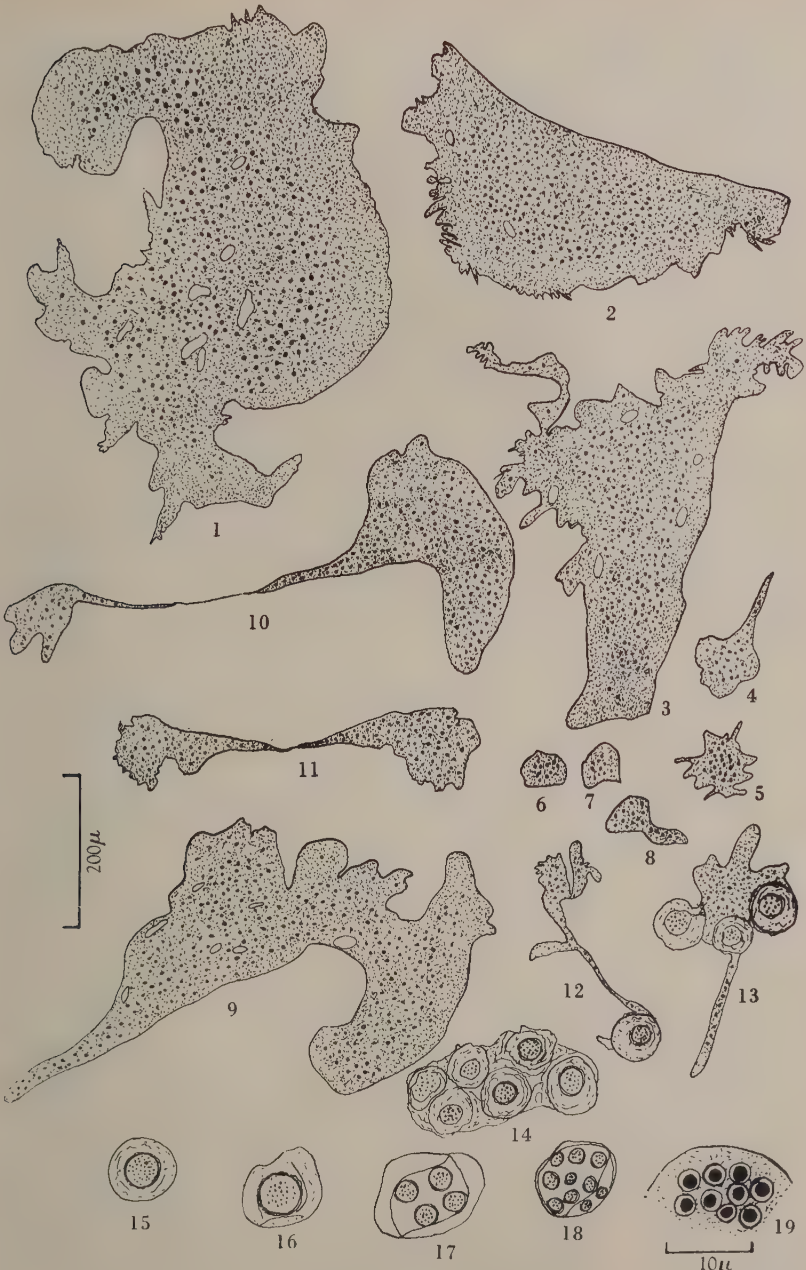
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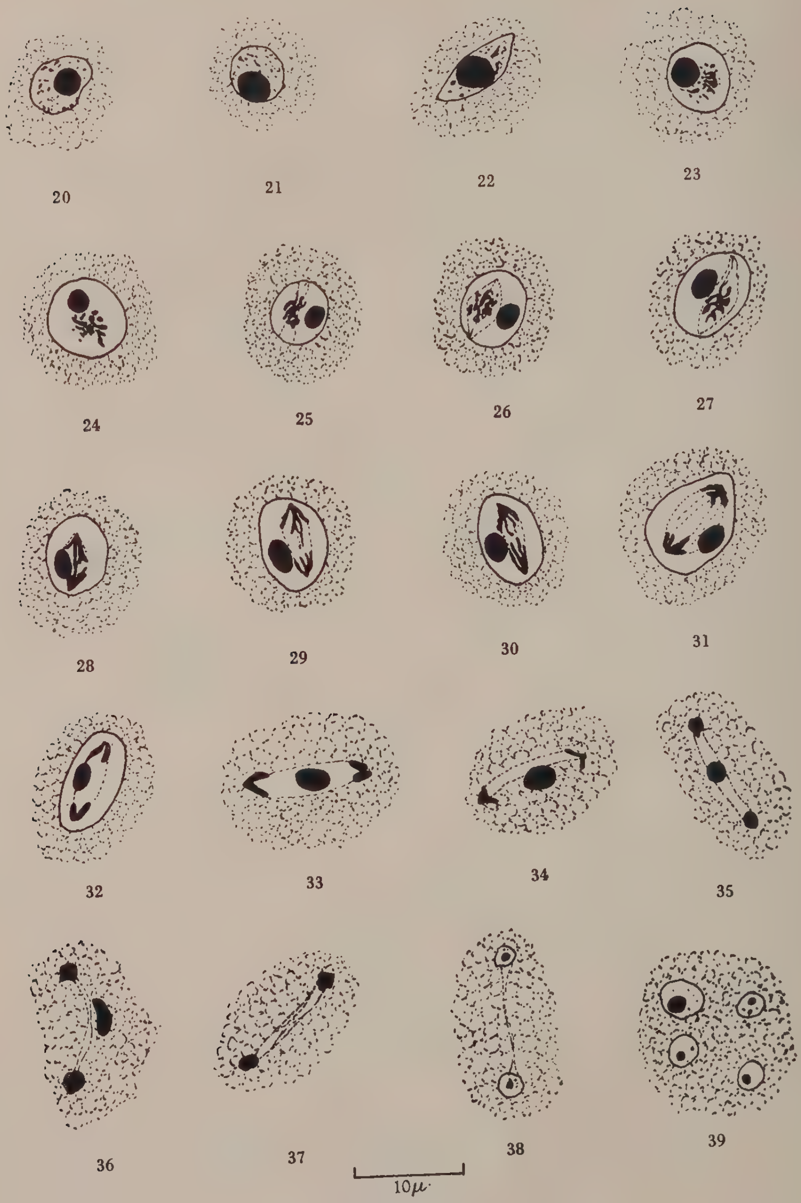
Figs. 1–19

B. N. Singh—Division and cyst formation in *L. reticulata*. Plate 1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Figs. 20–39

B. N. Singh—Division and cyst formation in *L. reticulata*. Plate 2

The Rules of Nomenclature for Micro-organisms

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The scientific names of organisms are the means by which one worker is able to relate his observations or opinions to those of others, and no one would benefit more than the microbiologist from an internationally acceptable and stable nomenclature. A large measure of agreement has already been reached on what constitutes an internationally acceptable name, but because appropriate and universally accepted names only reflect an agreed classification it will require much work by both systematists and experimental microbiologists before stability can be approached in many groups of micro-organisms. As a first step towards the goal microbiologists are urged only to use names which conform to international agreements because such usage makes for precision and at least does not hinder developments in classification. The purpose of this article is to advocate care in the use of names and to draw the attention of those who may wish to propose new taxonomic groupings or to change old names to certain requirements of the International Rules of Nomenclature. Also, systematists frequently feel obliged to make changes in the names of the groups of organisms which they study; changes which may at times seem arbitrary and unnecessary. A knowledge of the International Rules often enables the reasons for such changes to be more fully appreciated and sometimes allows a distinction to be made between name changes which are obligatory on all and those which need not be accepted at once, if ever, when they would disturb some long-established usage.

The International Rules

The names of animals are governed by the *International Rules of Zoological Nomenclature*, those of plants by the *International Rules of Botanical Nomenclature*. In 1930 the International Society for Microbiology at its first International Congress 'recognized that *insofar as they may be applicable* the nomenclatural codes agreed upon by the International Congresses of Botany and Zoology should be followed in the naming of microorganisms'. Fungi have been traditionally associated with plants, and mycologists accept the botanical code which includes several special clauses for their convenience. In 1930 the International Society for Microbiology also decided, in view of 'the peculiarly independent course of development' of bacteriology, that bacteriological nomenclature merited special treatment, and to that end set up an International Committee on Bacterial Nomenclature; later in the same year the two permanent secretaries of that committee being accepted by the Fourth International Botanical Congress as its Special Committee for Bacteria to make recommendations regarding bacterial nomenclature. In 1939 the International Committee on Bacterial Nomenclature was instructed to prepare a 'Bacteriological Code', and the publication of the code, when developed, was authorized by Congress on the understanding that it should be regarded as wholly tentative but in the hope that it would be widely tested. The war intervened, and it was not until the Fourth International Microbiological Congress in 1947 that a proposed code was approved. This tentative Code, which emphasizes the interdependence of bacterial and botanical nomenclature, is essentially an abbreviation and rearrangement of the Botanical Rules for bacteriologists, and in what follows any provisions of the Rules of Botanical Nomenclature applicable to bacteria have been tentatively accepted by bacteriologists.

Similarities between the International Rules of Botanical and Zoological Nomenclature

The zoological and botanical codes developed independently and each formally recognizes its independence: 'the name of an animal is not to be rejected simply because it is the name of a plant' (Zoological Rules, Article 1); 'the name of a plant is not rejected simply because it is the name of an animal' (Botanical Rules, Article 6). (Microbiologists, however, do not permit the use of duplicate generic names in the group *Protista* and deprecate the use of a specific name both in the *Protista* and in a plant or animal group; *J. Bact.* **33**, 447, 1937.) In spite of this independence the two codes have much in common. Both accept Latin binomial names for species, state or imply that every species has but one valid name in any genus in which it may be classified, judge the validity of names by the 'law of priority', and determine the application of names of species, genera and other taxonomic groups by means of 'types'.

Differences between the Rules of Botanical and Zoological Nomenclature

The Botanical Rules, which attempt to prescribe procedure for any eventuality, consist of 74 Articles and 50 numbered Recommendations. The first 19 Articles deal with general principles, the remainder are the 'Rules'. The Recommendations supplement and amplify the Rules, and many botanists appear to consider certain of the Recommendations to be as binding as Rules. The Zoological Rules are more concise and comprise 36 Articles together with recommendations for the formation of new names and the choice of types. There have been few changes in the Zoological Rules since they were first instituted in 1901, but the International Zoological Congress held in Boston in 1901 empowered the International Commission on Zoological Nomenclature, the body which prepared the Rules, to render 'Opinions' on questions of their interpretation. So far nearly 200 Opinions have been given, and these are in effect an official supplement to the latest, 1913, version of the Rules. A Judicial Commission, similar to the International Commission of the Zoologists, is proposed for the new Bacteriological Code.

The debt which biologists owe to Linneus for introducing binomial nomenclature and a tribute to his outstanding ability as a taxonomist are acknowledged by both codes in the starting-points selected for international nomenclature. The tenth edition of Linné's *Systema Naturae*, 1758, is the single starting-point for zoological nomenclature. Botanists accept the first edition of his *Species Plantarum*, 1753, as the starting-point for the nomenclature of flowering plants and some other groups including Lichenes and Mycetozoa, and it has been agreed that this shall also be the starting-point for bacterial nomenclature. For certain groups which were inadequately treated by Linneus, botanists have selected a number of later starting-points. The nomenclature of Fungi, for example, begins at 1801 (Persoon, *Synopsis methodica fungorum*) for Uredinales, Ustilaginales, and Gasteromycetes and at 1821–32 (Fries, *Systema mycologicum*) for other fungi.

Both botanists and zoologists at times find that the strict application of the law of priority would, by disturbing long-established usage, make for greater confusion than order and each code has its own method for providing alleviation in such cases. An International Botanical Congress may 'conserve' a later generic name against an earlier one and the International Commission on Zoological Nomenclature may 'suspend the Rules in any given case, where in its judgement the strict application of the Rules will clearly result in greater confusion than uniformity', and a Judicial Commission will have the power to proscribe names under the Bacteriological Code. Under no circumstances is it at present possible to uphold a later specific epithet against an earlier one which does not infringe the Rules.

In addition to the above differences there is a number of minor and more technical, although not unimportant, differences between the two sets of Rules, and attention will later be drawn to certain of them which reflect differences in current botanical and zoological taxonomic practices.

New species

An author wishing to propose a new species has first to satisfy himself that it is not one of the perhaps thousands of allied species already proposed and then to describe its characteristics so that they are recognizable to others. This is not the place to discuss the taxonomic question as to what constitutes a good species in any particular group, but to indicate the requirements of the International Rules if a new name is to prove acceptable.

The name must be in Latin and be in binomial form. The generic name should be spelt with a capital initial letter and that of the specific epithet should have no capital. It has never been obligatory to use capitals for specific epithets. Under the Zoological Code there is a Rule and under the Botanical Code a Recommendation that certain classes of specific epithets may be spelt with capital letters, and this practice has in the past been more widely followed by botanists than by zoologists.

Botanists are recommended 'not to make names long and difficult to pronounce', and both sets of Rules offer advice on what constitutes good taste in the selection and spelling of names. The name and the accompanying description must be published (for the conditions of effective publication see Botanical Rules, Art. 36), and under the Botanical Rules 'after 1 January 1935, names of new groups of recent plants, the Bacteria excepted, are considered as validly published only when accompanied by a Latin diagnosis' (Art. 38). The Zoological Rules do not insist on a Latin diagnosis, but the

Appendix to the Rules states that 'it is very desirable that the proposition of every new systematic group should be accompanied by a diagnosis, both individual and differential, of the said group in English, French, German, Italian, or Latin'. The same Appendix also advocates the use of the metric system of weights and measures in all descriptions and the expression of the enlargement or reduction of any illustrations in figures rather than by the system of lenses used.

The applications of the names of all taxonomic groups are determined by 'types', i.e. a family is based on a type genus, a genus on a type species, and a species or group of subspecific rank on a type specimen. Authors must always designate clearly the type on which their new group is based. When proposing a new species a zoologist is recommended to designate and label one specimen as the *type*, any other specimens examined by the author at the same time being *paratypes*. The types of species of fungi and bacteria may be descriptions with or without drawings or microscopical preparations. For bacterial species the type specimens are frequently cultures (when subcultures should be deposited in the principal culture collections of the world) and for microfungi they are commonly herbarium specimens. When a dried specimen, the type is frequently a 'collection' rather than an individual and it is probable that all the different parts of a single collection when deposited in several herbaria should be considered of equal status. If the type of a fungus is a culture, well-grown and typical cultures should whenever possible be dried as herbarium specimens, for in such form they are likely to be as much, or greater, use to future systematists as a living culture after a lengthy sojourn in a culture collection.

The account of a new species should always include the date of collection or isolation, the place of origin, and the host or substratum, if any, of the type material, and the location of the type specimen.

Name changes

'A generic or specific name, once published, cannot be rejected, even by its author, because of inappropriateness' (Zoological Rules, Art. 32). 'A name or epithet must not be rejected, changed or modified, merely because it is badly chosen, or disagreeable, or because another is preferable or better known' (Botanical Rules, Art. 59). Once a name has been validly published it cannot be eliminated. Even if reduced to synonymy it remains permanently as a name for the potential consideration of later workers. The Rules only permit the correction of a typographic error or a clearly unintentional orthographic error in the original spelling.

Names of species may, however, be changed for a number of reasons. The name may not be the earliest one, it may be a later homonym (i.e. the same name may have been applied previously to a different type), or, most commonly, it may need to be changed to accord with an author's views on classification. Under the Botanical Rules a change might also be necessitated because of tautonomy (i.e. because the specific epithet is an exact repetition of the generic name), but the Zoological Rules allow the use of tautonyms. If the new Bacteriological Code's acceptance of the botanical ruling is upheld such names as *Fusiformis fusiformis* Topley & Wilson will become illegitimate.

The valid names for fungi which have perfect and imperfect spore forms are the earliest names given to the perfect states; names given to other states having only a temporary value (Botanical Rules, Art. 57). The discovery of the ascospore- or basidiospore-producing state of a fungus of which the conidial state only is known involves, therefore, a change in the name of the latter according to the strict interpretation of the Rules, although when the perfect state is rarely encountered it is convenient and customary to continue to use the imperfect name.

Both Codes require the authors' names, usually abbreviated, to be added to all formal citations of the names of the taxonomic groups which they have proposed. Changes in the generic names of species involve changes in the authority for the name. Under the Botanical Rules, when a species is transferred from one genus to another the name of the author of the original species is enclosed in brackets and followed by the name of the author making the change. Under the Zoological Rules the first author's name is enclosed in brackets but the second author's name is omitted. Similarly, double citation is required for changes in rank of botanical names. For example, *Psalliota* (Fr.) Quél. indicates that *Psalliota* was proposed as the name of a subgenus by Fries and first used as a generic name by Quélet. Zoologists would not consider such a change to be a change in rank, for they treat genera and subgenera as equivalent. Neither do they distinguish a difference in rank from the nomenclatural point of view between subspecies and species. This difference reflects the greater stability of botanical genera and accounts for another contrast between the two disciplines. In botany the name of a species is the complete binomial, e.g. *Psalliota campestris*, which comprises the generic name *Psalliota* and the 'specific epithet' *campestris*. In zoology the stress is on the specific epithet, which is known as the 'trivial name' of the species, the combination of a trivial name with an appropriate generic name constituting the 'designation' of the species. When considering questions of priority among specific homonyms in zoology it is the one with the earliest trivial which is accepted, while in botany it is the earliest combination (binomial) which must be maintained, even though it contains a later specific epithet than the rejected name.

Synonyms

Synonyms are different names applied to one taxonomic group, but current practice restricts the application of the term to rejected names. Since the introduction of the type method there are two distinct classes of specific synonyms, those which are synonyms by definition and those which are matters of opinion. *Bacterium coli* (Mig.) Lehm. & Neum. and *Escherichia coli* (Mig.) Castel. & Chalm. are synonyms of the first kind. Both names are based on the same type and must therefore be synonymous. Both names are equally valid, and which is adopted depends on whether the user considers that the species should be classified in the genus *Bacterium* as proposed by Lehmann & Neumann or in the genus *Escherichia* of Castellani & Chalmers.

Bacillus vesiculi formans Henrici is listed in Bergey's *Manual* as a synonym of *Escherichia coli*, but this synonymy is a matter of opinion and not of definition. A second type is involved, and anyone is always at liberty to believe that the judgement of the authors of Bergey's *Manual* is at fault and that *Bacillus vesiculi formans* is the name of a distinct species. It is an aid to clear thinking if these two classes of synonyms are distinguished when citing synonymies, and this may be conveniently done by adding the name of the author whose judgement is accepted as the authority for a synonym of the second class, e.g. *Escherichia coli* (Mig.) Castel. & Chalm., syn. *Bacillus vesiculi formans* fide Bergey *et al.*

LITERATURE

The current official version of the *International Rules of Zoological Nomenclature*, that agreed on by the International Zoological Congress of 1913, is out of print. The Rules have, however, been reprinted in C. M. Wenyon, *Protozoology* (1926) **2**, 1936 (London) and in *Proc. biol. Soc. Wash.* (1926) **39**, 75 (together with summaries of the first 90 Opinions), and in the useful book by E. T. Schenk & J. H. Masters (1936), *Procedure in Taxonomy* (U.S.A. and Oxford Univ. Press, London). The 194 'Opinions and Declarations rendered by the International Commission on Zoological Nomenclature' have recently been published or are being republished in three volumes by the Commission (London), and in 1943 the same Commission instituted *The Bulletin of Zoological Nomenclature* in which proposals submitted to the Commission for deliberation and decision are printed. In the first issue of that *Bulletin* (Vol. **1**, p. iv) there is an interesting article by F. Hemming on the functions and powers of the International Commission.

The official French, English and German versions of the *International Rules of Botanical Nomenclature* were issued in 1935 (Gustav Fischer, Jena). The Rules have been reprinted (without the Appendices) in C. W. Dodge, *Medical Mycology*, pp. 76–96 (U.S.A., 1935; London, 1936) and (abbreviated) in Bergey's *Manual of Determinative Bacteriology*, 1939, 5th ed. They have also been reprinted and annotated for mycologists in G. R. Bisby, *An Introduction to the Taxonomy and Nomenclature of Fungi*, 1945 (Imperial Mycological Institute, Kew), a book which gives much useful information on procedure.

The *Proposed Bacteriological Code of Nomenclature*, edited by R. E. Buchanan and R. St John-Brooks, 1947 (Iowa State College Press), will be published in the *Proceedings* of the Fourth International Congress for Microbiology. It gives, in addition to the proposed rules, a historical account of their development and the opinions issued by the International Committee on Bacteriological Nomenclature and ratified by Congress during the period 1930–9.

'A discussion on the differences in observance between zoological and botanical nomenclature', *Proc. Linn. Soc. Lond.* (1944) **156**, 126 and 'Discussion on mycological nomenclature', *Trans. Brit. mycol. Soc.* (1942) **25**, 428, provide clear expositions of many points of detail.

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Infections by *Leishmania donovani* in the Cotton Rat

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SUMMARY: Following the original inoculation of cotton rats with material from the spleen of a golden hamster containing Leishman-Donovan bodies, a number of serial passages were subsequently carried out in these animals. The parasite became rapidly adapted to the new host and a progressive infection resulted comparable with that in the golden hamster. Whereas death in the latter animal is the invariable sequel of infection, the cotton rat appears to be unaffected by the disease. The cotton rat thus provides a new and easily obtainable host for the study of experimental leishmaniasis in this country, including the chemotherapy of the disease.

Most laboratory animals are unsuitable as hosts for the study of experimental leishmaniasis on account of the irregularity with which infection occurs and the prolonged incubation periods encountered in successful cases. Infections, moreover, frequently undergo spontaneous cure. Smyly & Young (1923–4*a*, *b*) in China found that the Chinese hamster (*Cricetulus griseus*) was highly susceptible to visceral leishmaniasis and failure to infect was rarely experienced. Mayer (1926) then found that the European hamster (*Cricetus frumentarius*) was equally susceptible to infection with an Indian strain of *Leishmania donovani*. Adler & Theodor (1931) infected the golden hamster (*Cricetus auratus*), a native of Syria, with *Leishmania infantum*. This host was later employed by Adler & Tchernomoretz (1939) in assessing the value of stilbamidine in *L. donovani* infections. Up to the present the golden hamster has been the only animal readily available in this country for such tests (Fulton, 1944; Goodwin, 1944). Besides being very susceptible to infection it is easily handled, but it breeds well in captivity only for part of the year. We have now found that the cotton rat (*Sigmodon hispidus hispidus*), which was recently introduced to this country for the study of rickettsial diseases, is also very susceptible to visceral leishmaniasis. The details of the initial experiments with this animal are recorded below.

METHODS

The infective material was obtained from the spleen of a hamster infected with a strain of *Leishmania donovani* that had been maintained in hamsters since its isolation from an Indian patient with kala-azar in 1939. After mincing with scissors, spleens were emulsified with pestle and mortar in Ringer glucose and passed through a thin layer of cotton-wool to remove gross particles. The suspension was in all cases injected intraperitoneally in 0·5 ml. amounts to animals approximately 6 weeks old. One heavily infected organ provided sufficient material to inoculate 20–30 animals and gave rise to infections which could be detected in spleen smears 2–3 weeks later. The number of Leishman-Donovan bodies in the inoculum was estimated either by counting the red cells present after suitable dilution, followed by relative counts of red cells and

Leishman-Donovan bodies in a stained film, or by mixing the initial suspension with one of fowl cells of known dilution and counting the relative numbers of red cells and parasites in a stained preparation. In order to determine the progress of infection in the experimental animals they were killed at intervals, and a rough count of the number of parasites was made in smears of liver and spleen under standard conditions with $\frac{1}{12}$ in. oil-immersion lens and 6 × eyepiece. The infection in spleen is accepted as a good indication of the degree of general infection. When stained smears from animals killed first in a series contained no detectable parasites, cultures were made of liver and spleen on Locke serum-agar medium.

RESULTS

In the first experiments with cotton rats four animals were inoculated at the same time as 40 hamsters with infective material from the spleen of one hamster. Forty-three days later, the hamsters underwent partial splenectomy for another purpose. The number of parasites in splenic smears from this batch varied from a few per 100 fields to 150 in a single field. In two cotton rats killed 65 days after inoculation liver and spleen smears contained only a few parasites per 100 microscopic fields, whereas in two cotton rats killed after 99 days, the counts were 40 per field, showing that the infection had progressed with time. In view of these results, serial passage was carried out at the same time in both cotton rats and hamsters. These passages, in which animals of both species were sometimes given the same inoculum and on other occasions were inoculated with infective material from their own species, are summarized in Fig. 1.

The details of the resulting infections are given in Table 1.

Passage A. Very heavy infections occurred in hamsters from the 56th day after inoculation onwards, seven animals dying on the days indicated. The cotton-rat infections were less heavy at the earliest examinations, but later were as intense as those in the hamsters. It is noteworthy that the health of cotton rats appeared to be unaffected. Three whole heavily infected hamster spleens were used to provide the infective material.

Passage B. The infective material was obtained from the spleens of two very heavily infected cotton-rats (index 4 and 5, see Table 1). In both types of animal the infection appears to have developed with equal rapidity. The series was, however, small, and it is well recognized that individual infections in a batch of hamsters receiving the same inoculum shows considerable variations in intensity.

Passage C. The cotton rats were inoculated with a moderately infected spleen (index 2) from an animal of the same species and the hamsters with a very heavily infected spleen (index 5). From previous experience with hamsters the heavier inoculum would be expected to give a shorter latent period of infection. This proved to be the case; in the cotton rats infection in smears was first detected 65 days after inoculation, as compared with 9 days in hamsters. In cotton rats, however, cultures revealed the presence of parasites as early as the 28th day. The final intensity of splenic infection was similar in both groups.

Passage D. Eighteen cotton rats were infected from the spleens of three animals of the same species with very heavy infections (index 5). Infection was detectable from the 17th day onwards and progressed till the 101st day when the remaining twelve animals were killed and all were found to have heavily infected spleens.

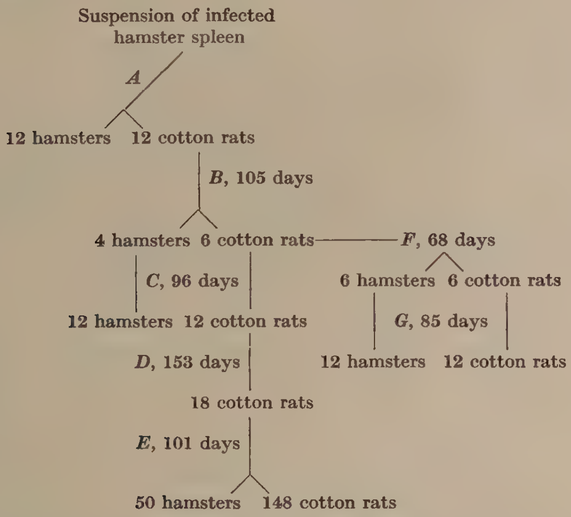
Suspension of infected hamster spleen
A
12 hamsters
12 cotton rats
B, 105 days
4 hamsters
6 cotton rats
F, 68 days
C, 96 days
6 hamsters
6 cotton rats
12 hamsters
12 cotton rats
G, 85 days
D, 153 days
12 hamsters
12 cotton rats
18 cotton rats
E, 101 days
50 hamsters
148 cotton rats

Fig. 1. The course of serial passage of leishmania in cotton rats and hamsters. The letters designate the passages made and the days refer to the interval between each passage and the preceding one.

Passage E. One hundred and forty-eight cotton rats and 50 hamsters were each inoculated with approximately 14 million parasites obtained from the spleens of 12 cotton rats with heavy infections (index 4 and 5); 10 animals were killed 14 days after inoculation and parasites were seen in the organs of six. Another 10 were killed on the 28th day after inoculation and parasites were found in all liver, spleen and bone-marrow smears. Similar results were obtained in a further 10 animals killed 42 days after inoculation. In the hamsters infection progressed in the same way. The experiment is still in progress.

Passage F. The spleen and liver of a cotton rat with a heavy infection (index 4) provided the inoculum. The progress of the disease was approximately the same in both batches of animals and caused the death of one hamster.

Passage G. Both cotton rats and hamsters were inoculated each with approximately 12 million Leishman-Donovan bodies from their own species. The course of the infection in both types of animal was very similar, though the hamsters began to succumb to heavy infections 71 days after inoculation, whereas cotton rats, with equally progressive heavy infections, survived and remained well; 248 days after inoculation four cotton rats were still in good health and when killed had very heavy liver and spleen infections (index 5), while that in bone marrow was less heavy (index 4–5). The spleens of these four animals had greatly increased in size, one weighing 800 mg. in contrast with the 70 mg. of a normal animal of the same age. The oedema in golden hamsters chronically infected with *L. donovani* described by Goodwin (1945) and the accompanying amyloidosis described by Gellhorn, van Dyke, Pyles & Tupikova (1946) was not seen in cotton rats with similar long-standing infections.

Table 1. *The comparative results of passage of* Leishmania donovani *in cotton rats and hamsters*

		Index of infection by Leishman-Donovan bodies.					
		Cotton rats			Hamsters		
Passage	No. of animal	Days after inoculation	Liver	Spleen	Days after inoculation	Liver	Spleen
A	1	26*	ne	ne	56†	ne	5
	2	81	3	2	87†	,,	5
	3	81	4	3	91†	,,	5
	4	81	3	3	94†	,,	5
	5	105	5	5	94†	,,	5
	6	105	3	4	95†	,,	5
	7	132	5	5	96	,,	5
	8	132	5	5	97	,,	5
	9	147	4	4	97	,,	5
	10	147	4	4	120†	,,	5
	11	161	5	5	125	,,	5
	12	161	5	5	125	,,	5
B	1	26	2	2	26	2	2
	2	42	4	3	42	2	4
	3	56	4	4	—	—	—
	4	69	5	4	—	—	—
	5	82	5	5	82	ne	3
	6	97	5	3	97	5	5
C	1	9	0	0	9	0	2
	2	21	0	0	21	0	1
	3	28	0	1	28	2	2
	4	35	1	0	35	2	2
	5	43	0	1	43	0	3
	6	55	0	1	55	2	4
	7	65	2	2	65	2	3
	8	77	1	1	77†	ne	4
	9	90	2	2	90	3	5
	10	153	5	5	94†	ne	5
	11	153	5	5	94†	,,	5
	12	153	5	5	100	,,	5
D	1	17	2	2	—	—	—
	2	17	1	2	—	—	—
	3	31	3	2	—	—	—
	4	31	2	2	—	—	—
	5	53	2	2	—	—	—
	6	53	3	4	—	—	—
	7–18	101	ne	4–5	—	—	—

Table 1 (*cont.*)

		Cotton rats			Hamsters		
Passage	No. of animal	Days after inoculation	Liver	Spleen	Days after inoculation	Liver	Spleen
F	1	22	3	3	22	2	3
	2	29	2	2	29	2	2
	3	37	2	1	37	2	3
	4	50	3	2	50	2	3
	5	71	2	3	64†	ne	5
	6	84	3	5	64	3	5
G	1	9	1	1	15	1	1
	2	12*	ne	ne	21	0	2
	3	15	0	1	30	0	2
	4	21	2	2	44	2	3
	5	30	3	2	66	3	5
	6	44	5	4	71*	ne	ne
	7	66	5	4	110†	,,	5
	8	114	4	5	111†	,,	5
	9	248	5	5	112	5	5
	10	248	5	5	117†	5	5
	11	248	5	5	118*	ne	ne
	12	248	5	5	123	5	5

Under 'Days after inoculation' † indicates death from leishmaniasis, and * death from unascertained cause.

Index of infection:

1 = smears negative, culture positive.
2 = 1–100 Leishman-Donovan bodies per 100 microscope fields.
3 = 101–1000 Leishman-Donovan bodies per 100 microscope fields.
4 = 1001–5000 Leishman-Donovan bodies per 100 microscope fields.
5 = > 5000 Leishman-Donovan bodies per 100 microscope fields.
ne = not examined.

During the course of these experiments two litters of rats were born of infected parents. The six young of one litter were killed when respectively 14, 33, 33, 34, 45 and 74 days old and the four young of the second litter when 2, 75, 113 and 113 days old. In none of these animals could infection be detected in smears or cultures of liver and spleen.

Attempts were also made to infect 12 Orkney voles (*Microtus orcadensis*) at the same time as cotton rats and hamsters. Examinations of liver and spleen smears were consistently negative and only one positive culture of spleen was obtained. The Orkney vole therefore seemed an unsuitable host for *Leishmania donovani* and the experiments with it were not pursued.

DISCUSSION

The golden hamster, as the only susceptible animal so far available in this country, has been almost exclusively used in the study of experimental kala-azar. Our results clearly show that the cotton rat is also a suitable host for this infection. In the earlier experiments the number of parasites used for inoculation was not determined, so it is not possible to say whether the infection has become more or less virulent for the new host. The organs of 98 cotton rats which received an infective inoculum have been examined. In eight of these examined within 28 days after inoculation, infection could not be demonstrated. Some of the failures were due to contamination of the cultures. Of the 95 hamsters inoculated at the same time only one which was examined 14 days after inoculation failed to show infection. In our experience with many hundreds of hamsters we have never failed to produce infection and we consider that such would have proved to be the case with the cotton rats had they been examined after a longer incubation period. The infection progresses in cotton rats as regularly as in hamsters with no tendency to spontaneous regression up to a period of 248 days after inoculation, and the presence of parasites in stained smears can generally first be detected after the same period in both hosts. The irregularities, well recognized in hamsters, in the development of infection in individual animals of a batch receiving the same inoculum also occurs in the cotton rat. The spleen of both hosts increases remarkably in size. The degree of general infection in the hamster is indicated by the number of parasites in the spleen (Adler & Tchernomoretz, 1939), and judging by the limited examinations of infected cotton rats this observation seems to hold also for these animals. In chemotherapeutic experiments some workers puncture the liver in preference to partial splenectomy in order to establish the presence of infection before treatment, although the mortality rate during this procedure is sometimes high. The liver in cotton rats during the early stages of infection is as heavily infected as the spleen, and liver puncture might therefore be used with greater advantage than in the case of hamsters, especially in view of the susceptibility of the former to poisoning by the common anaesthetics. It is of interest that the cotton rat, like the Chinese hamster, appears to be unaffected in health by the heaviest infections, in marked contrast to the golden hamster, in which death is the invariable sequel. Though the cotton rat is less easily handled than the hamster it is much bigger when fully grown and thus possesses certain advantages for special purposes. It is also a prolific breeder all the year round, whereas the breeding season of the hamster is limited. We have not so far used the cotton rat extensively in chemotherapeutic experiments and its value in this type of investigation has still to be determined. In view of the different results obtained by Wang (1939) on the one hand, using the Chinese hamster, and by Kikuth & Schmidt (1938), with the European hamster, on the other in the treatment of kala-azar with solustibosan, similar differences may be expected when this new host is employed. Moreover, Cowie & Lawton (1947) have recently demonstrated that the fate of an antimonial drug may vary widely with the species of animal used. It is still problematical how far the use of the cotton rat will serve as a guide to the treatment of the human disease, since in addition the pathogenicity of *L. donovani* varies widely in the different hosts.

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The Inhibition of *Fusarium oxysporum* var. *cubense* by Musarin, an Antibiotic produced by Meredith's Actinomycete

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SUMMARY: Under selected conditions Meredith's actinomycete produced culture fluids toxic to many fungi. From these fluids the active principle, called musarin, has been isolated by two methods. Preparations active in concentrations of 1/80,000–1/100,000 seemed substantially pure.

Musarin is an optically active acid of high molecular weight having by microtitration an equivalent weight of at least 4000. Analyses of the best preparations are in good agreement with an empirical formula $(C_{35}H_{60}O_{14}N_2)_{72}$. No definite conclusion can be drawn about the exact chemical nature of musarin; a protein-like structure seems to be excluded by the low nitrogen content, which recalls that of many bacterial polysaccharides; but formulation as a polysaccharide seems equally unsatisfactory because of the low oxygen content.

Tests indicate that musarin is one of the most potent antifungal antibiotics, though as an antibacterial agent it is less effective. It is active against several important plant parasites and it may be useful in combating the Panama disease of the banana plant and other fungal diseases of economic importance.

The so-called Panama disease of the banana plant (*Musa sapientum*), caused by *Fusarium oxysporum* var. *cubense*, is known to be inhibited by certain Jamaican soils. From such soils Meredith (1943, 1944) isolated actinomycetes which were able to inhibit or retard the development of *F. oxysporum* var. *cubense*. Later it was shown that Meredith's actinomycete produced soluble antibiotics against *F. oxysporum*, particularly when grown on food yeast waste liquor (Thaysen & Butlin, 1945) or on meat extract media (Thaysen & Morris, 1947).

Whilst the biological aspects of the problem are being further investigated by Dr A. C. Thaysen in the Colonial Microbiological Research Institute, Trinidad, we record here a study of the production, isolation and properties of a new antibiotic obtained from the culture filtrate of the actinomycete, and termed for convenience *musarin*. This name, although recalling the host plant rather than the parent organism, seems justified as it avoids confusion with antibiotics derived from other actinomycetes.

EXPERIMENTAL

Cultural characters of Meredith's actinomycete

The original culture of Meredith's actinomycete grown on peptone or synthetic agar media was dry, wrinkled and uniformly white, but on malt-extract agar the cultures became deeply pigmented, pale to deep yellow, red, green and a few chalk-white colonies appearing in a single culture. When malt-agar plates were inoculated from these variously pigmented growths it was found that all the young colonies were white or yellow, the red and green pigments developing later in some of the yellow colonies. Green colonies often became dark red, almost black, after several days; but some colonies started turning red on the third day of growth. By selection and successive replating of the earliest red colonies a culture in which every colony turned red was finally obtained. By similar selection, homogeneous chalk-white and deep-yellow cultures were also obtained; but since the red strain was found to give the best results the other strains were not investigated in detail. The red strain was, however, unstable and had to be continuously selected. Moreover, the colour of the colonies was not a sure indication of the capacity to produce active solutions when grown on liquid media and the above relationship of colour and activity changed after repeated transfers. It was noticed, however, that active strains invariably produced a dark, diffusible pigment on both malt-agar and in liquid casein media, although the pigment had no other connexion with the active principle; musarin itself is colourless.

Methods of testing for antibiotic activity

For the routine examination of actinomyces culture liquids for antifungal activity *F. lateritium* was used as the test organism in preference to *F. oxysporum* var. *cubense*, since it readily produces masses of spores.

(*a*) *Agar-plate method.* Malt-extract agar (for fungi) or peptone Lemco agar (for bacteria) was seeded with the test organism and plates were poured. The antibiotic liquids were filled into holes cut in the medium with a cork-borer 1 cm. diameter. The width of the inhibition zone was read after overnight incubation at 37° for bacterial plates. Fungal plates were incubated at 24°, and 2 or occasionally 3 days' incubation was necessary before results were obtained. The width of the zone varied with potency of the fluid. A second, and sometimes a third, ring or partial inhibition usually developed beyond the completely clear zone, frequently edged with a narrow ring of stimulated growth.

(*b*) *A serial broth dilution method* was also employed for antibacterial tests.

(*c*) *Hanging-drop method for anti-spore germination tests.* A suspension of *Fusarium* spores in nutrient broth was mixed with an equal quantity of solution or with serial dilutions of antibiotic liquids and hanging-drop slides were prepared from the mixtures. Results were read after overnight incubation at 24°. The antifungal titre of the solution was taken as the highest dilution completely inhibiting spore germination (+ + + +). One unit of antibiotic potency is arbitrarily defined as the amount of musarin which in 1 ml. of nutrient medium completely inhibits *F. lateritium* spore germination. The following degrees of partial inhibition were also noted: (1) emergence of germ tubes from some of the spores but no further growth (+ + +); (2) germination of the spores but no normal mycelial growth, yeast-like budding (+ +); (3) limited mycelial growth followed by formation of masses of microconidia (+).

The width of the inhibition zone obtained by method (*a*) was closely correlated with the degree of inhibition obtained by method (*c*) in parallel tests of the same culture filtrate. Roughly, +, + +, + + +, + + + + corresponded to titres of *c.* 1/2, 1/4, 1/8 and 1/16 respectively, as estimated by method (*d*).

(*d*) *Dilution plates for tests of antifungal activity.* Solutions of musarin were added to measured quantities of melted malt-extract agar to give final dilutions of 1/50,000, 1/100,000, etc., and the mixture was poured into Petri dishes. A loopful of a spore suspension of the fungus was placed on the surface of each dilution plate. The plates were incubated at 24° and examined for spore germination and mycelial growth at intervals up to 6 days. In most cases there was good mycelial growth on the control plate after 40 hr. In the antifungal tests the results given by methods (*a*) and (*d*) and in a limited number of tests by method (*c*), were closely correlated.

Production of musarin

Musarin was detected by its antibiotic activity against *F. lateritium* spores, tested as described above. It was produced when the actinomycete was grown in stationary, or shaken (submerged) cultures (see below); it is possible that under different conditions more than one antibiotic was formed but the stability and biological properties of the active culture fluids suggested that the antibiotics were at least very similar.

Stationary cultures. The actinomycete was grown in 150 ml. conical flasks containing 40 ml. of a defined medium, consisting of KCl 0·5 g., $MgSO_4.7H_2O$ 0·5 g., $FeSO_4.7H_2O$ 0·01 g., and K_2HPO_4 1·0 g., in 1 l. of water to which was added yeast extract, malt extract, Eupepton No. 2, Bacto-Tryptone or casein, either with or without 1 % glucose. With the exception of the yeast-extract medium without sugar and the malt-extract medium with sugar, cultures in all the media were active against *F. lateritium*. The greatest activity was obtained when 1 % glucose and either 0·5 % casein or 0·5 % Eupepton were added. Using the original mixed actinomycete culture, the fluid after 12 days at 25° contained 8 units/ml. with strong retardation of mycelial growth. It also completely inhibited the growth of *Mycobacterium phlei* at 1/50, and was fairly active against *Staphylococcus aureus* and *Bacillus subtilis*, but only feebly active against *Bacterium coli* and *Pseudomonas pyocyanea* (*Ps. aeruginosa*).

In one experiment, a flask was coated on the inside with a thin layer of malt-extract agar, and 40 ml. of Eupepton-glucose solution were added after the agar had set. The actinomycete grew well in the solution and formed a thick ring of growth on the agar at the surface of the fluid, which became deeply pigmented. This culture contained 8 units/ml. but caused slight inhibition at 1/64, and a very definite clear zone, 8 mm. wide, on a malt-agar plate seeded with *Fusarium lateritium* (method *a*). Similar results were obtained on Lemco peptone-agar slopes with solutions of 1 % glucose, lactose or sucrose broth at the bottom of the tubes, the sucrose culture being the most active.

When four flasks were inoculated respectively with the red, yellow and white strains and the original (mixed) culture, the medium containing NaCl 5 g., K_2HPO_4 1·0 g., glucose 10 g., and casein 5 g., in 1 l., all except the yellow strain produced dark brown pigment. Antibiotics against *F. lateritium* spores appeared most rapidly (11 days) in the red culture, slowly in the original and white strains and very slowly in the yellow, but the final maximum titre was the same in all four cultures. All four cultures were equally active against *Myco. phlei* on the 20th day (1/25) but were inactive against *Bact. coli*. The white culture alone feebly inhibited *Streptococcus pyogenes* and was also slightly more active than the other three against *Staph. aureus* and *B. subtilis*.

In a second series of tests the red, white and yellow strains were inoculated into flasks, the interior of which had been coated with Lemco peptone-agar, containing 18 ml. of liquid casein-glucose medium. Again the red strain produced dark brown pigmentation of both the agar and the liquid but the yellow culture did not do so; the white culture slowly produced pigmentation of the agar but the liquid remained uncoloured. As before the red culture produced the antibiotic most rapidly, though on this occasion the white was slower than the yellow strain. In general, activities against a variety of organisms were similar to those in the previous experiment except that the red cultures were active against *Bact. coli* whereas the white and yellow strains were inactive; moreover, the red strain was as active as the white against *Strep. pyogenes* whereas the yellow was inactive.

All three cultures were very active against *Verticillium albo-atrum* and *V. dahliae*; the diameter of the inhibition zone (method *a*) on malt-agar plates was 18 mm.

Production in quart-size milk bottles containing 400 ml. of medium was unsuccessful. With a medium similar to that used for the production of streptothricin by *Streptomyces lavendulae* (Waksman, 1943), containing K_2HPO_4 0·5 g., NaCl 0·5 g., $FeSO_4.7H_2O$ 0·01 g., glucose 10 g., and Bacto-Tryptone 5 g. in 1 l. of water, antibiotic was produced only when the organism could be induced to grow on the surface by providing support in the form of pieces of porous porcelain. With a starch (2 %) glycine (1 %) medium (Waksman, 1943) no antibiotic was obtained, probably for the same reason.

The active fluid from stationary cultures was unaffected by acid (pH 2·4) or alkali (pH 10·5) at room temperature for 30 min. but was completely inactivated at 100°.

Shaken cultures. Shaken cultures of the above Bacto-Tryptone glucose medium, using the original culture of the actinomycete, produced antibiotic after 14 days (2 units/ml.). Eupepton, glycine and corn-steep liquor were less effective in that order. The antibiotic so produced was as stable as that in stationary culture fluids. Heating at 100° at pH 7 for 30 min. inactivated 50 %.

Submerged cultures. The fermentation vessel (Fig. 1) was a tank of ¼ in. plate iron 15 in. high and 16 in. diameter, designed so that when charged with medium and assembled the whole could be sterilized as a unit. It was fitted with a stout screw-on iron lid and rubber washer; the lid was removable for cleaning but carried a screw-on cap (*C*) for charging, inoculating, and sampling, and a metal stirrer (*S*) the paddles of which closely fitted the vessel. The tank was provided with an inlet (*I*) connected to a circular iron tube drilled in the form of a gas-ring burner and reaching almost to the bottom of the tank. When in operation the stirrer was mechanically driven and sterilized air was injected through the inlet *I* by a pump so that the medium was thoroughly aerated. The tank was used about half-full with a charge of about 20 l. No special temperature control was necessary, but good results were obtained only when the room temperature was 20–25°. With a small excess pressure inside the apparatus and a tight-fitting stirrer gland and cotton-wool packing at *C* during sterilization, no contamination occurred. The iron scaled during use; this was not harmful, and plating the tank with stainless steel had no marked advantage.

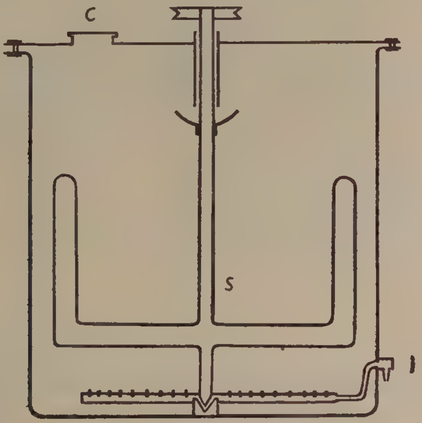

Fig. 1. Fermentation vessel.

Inoculations were made with a single-slope culture of actinomycete, previously macerated with water. Rapid fermentation, especially in presence of higher concentrations of Bacto-Tryptone, caused much inconvenient frothing through the loosely fitting sampling lid *C*. The concentration of Bacto-Tryptone, which seemed to be the important nutrient for antibiotic production, was therefore chosen so that fermentation and antibiotic production took about 4 days. More rapid production was followed by more rapid destruction of the antibiotic by aeration and it was found impracticable in view of the methods of assay to estimate the time of peak activity in such rapid fermentations. It was also found advantageous to decrease the rate of aeration to the minimum and to limit the amount of medium to about 17 l. in order to avoid frothing.

Submerged cultures of the original mixed strain were grown in the tank using a medium containing NaCl 0·05 %, K_2HPO_4 0·05 %, $FeSO_4.7H_2O$ 0·0025 %, Eupepton 0·25 %, Bacto-Tryptone 0·25 %, and glucose 1 %. After 4–5 days the solution contained 32 units/ml., but after 6 days the potency had dropped to 8 units/ml. With 1 % Bacto-Tryptone fermentation was very rapid and although the culture was slightly active after 2 days, the activity soon disappeared. When Bacto-Tryptone was replaced by Eupepton the maximum production of antibiotic was 2 units/ml.

The red strain of the actinomycete was found to produce as much antibiotic as the mixed culture, but the yellow strain produced little or none with either Bacto-Tryptone (0·5 %) or Bacto-Tryptone (0·25 %) and Eupepton (0·25 %) (max. 2 units/ml.). Growth of the white strain on the standard medium (Bacto-Tryptone 0·25 %, Eupepton 0·25 %) was slower than with the red; its cultures consistently contained 16 units/ml. after 4 days, dropping to 8 units/ml. after 5 days. The red strain, giving 32–64 units/ml., was therefore selected for further study. The effect of changing some of the constituents of the standard medium is shown in Table 1, which indicates that the standard medium had the most favourable composition for antibiotic production by the red strain.

Table 1. *Effect of variation of medium on the antibiotic production by the red strain of Meredith's actinomycete*

Component varied	Potency (units/ml.)
Bacto-Tryptone (0·375 %)	32–80
Bacto-Tryptone (0·25 %) Eupepton nil	12
Bacto-Tryptone (0·375 %) Eupepton (0·125 %)	32–64
Glucose (1·5 %)	2
Standard medium	32–64

Isolation of musarin from the fermented tank solution

Early experiments with stationary culture fluids showed that the only solvent suitable for extracting musarin was *n*-butanol at pH 7. Extraction of a shaken culture by this means removed the antibiotic activity completely. The butanol layer was separated, concentrated *in vacuo*, and treated with excess of ether, when an almost white solid precipitated. The solid was filtered off; it was slightly hygroscopic and turned brown in air. This material contained 35 units/mg. This method failed with large-scale extractions because the antibiotic produced by submerged fermentation was thermolabile. The thermolability proved to be due to the impurities in the culture fluid, since purified preparations were found to be relatively thermostable. It was hoped to back-extract the active material into water by adding ether (5 vol.) or light petroleum (5 vol.) but the partition coefficients were unfavourable, only 66 and 50 %, respectively, of the activity being recovered in the aqueous layer. The antibiotic was adsorbed on charcoal (2 %) and partially on fuller's earth (1 %) but could not be satisfactorily eluted. In this respect, as in many others to be described later, musarin differs from streptomycin and streptothricin, which can be eluted from charcoal with acid alcohol (Le Page & Campbell, 1946; Peck, Walti, Grater, Flynn, Hoffhine, Allfrey & Folkers, 1946). Experiments with precipitating reagents showed that musarin was precipitated by adding ammonium sulphate almost to saturation; 64 % of the activity could be recovered from the precipitate by extraction with water and only 47 % by extraction with methanol. Musarin was partially precipitated from the culture fluid by NaCl, but not by $Ca_3(PO_4)_2$. The addition of acid to pH 3 also precipitated most of the active material. Two methods of isolating musarin are described here.

(1) To 20 l. culture filtrate at 0° containing 160,000 units was added 9 kg. ammonium sulphate. The solution was allowed to stand for 2 hr. and the precipitate (A) was filtered off; on further standing more solid (B) precipitated. A cold methanol extract of precipitate A, after concentration *in vacuo* and precipitation with ether, yielded 933 mg. of a white solid (20 units/mg.; 18,500 units). A second extraction with hot methanol yielded 1178 mg. of a solid containing 47,100 units. Precipitate B (10 g.; 100,000 units), treated in a similar way, yielded 187 mg. (7000 units) and 239 mg. (2400 units) respectively. The total amount of musarin recovered was therefore 75,000 units, corresponding to a recovery of 47 %. In addition, 203 mg. of a solid containing 16,000 units were obtained from the air-dried mycelium by methanol extraction. An aqueous solution of any of these materials gave a precipitate with mercuric chloride or copper acetate solution but no active fraction was recovered. Solution of the less active solids in 1 % neutral potassium phosphate buffer and addition of 2 N-H_2SO_4 to pH 3 gave a precipitate, which was separated by centrifuging and washed with acetone and ether. This solid, which decomposed at about 170°, contained 80 units/mg. It contained neither halogen nor sulphur; it was insoluble in water but dissolved readily in 1 % neutral phosphate buffer.

(2) The following method gave better results and was used for routine isolation of musarin.

The tank culture fluid was freed from all solid matter by decanting and filtration. The filtrate (15 l., containing 480,000 units) was acidified to pH 3·5–4·0 with dilute H_3PO_4. The addition of *c.* 0·5 % neutral alumina or silica before acidification at this point often facilitated the subsequent filtration but was not essential. A precipitate appeared almost immediately and was allowed to settle. The solution was then filtered and the filtrate, containing 60,000 units, was discarded. The precipitate was eluted by shaking vigorously for 1 hr. with 1·6 l. 1 % neutral sodium phosphate buffer solution. The eluate was filtered (320,000 units; recovery, 67 %) and extracted twice with 500 ml. *n*-butanol. After the first extraction 32,000 units (10 %) and after the second extraction only 3200 units (1 %) remained in the aqueous layer. The butanol extracts were combined, washed with a little water, and evaporated to dryness *in vacuo* at 45°. The residue was dissolved in *c.* 100 ml. of methanol and the solution filtered and concentrated to small volume *in vacuo*. Addition of ether precipitated 2 g. of a slightly yellow solid, containing 100 units/mg. The recovery was therefore at least 200,000 units or 42 %. Some of the yellow pigment could be removed by reprecipitating the antibiotic from methanol with acetone, but no increase in potency was achieved by this treatment.

The mycelium of the actinomycete also yielded musarin, though the quantity isolated was somewhat variable and the potency was usually low. In one instance the air-dried mycelium from 20 l. of fermented solution was extracted with hot methanol (wt. after extraction = 7·7 g.). The solution was filtered and evaporated to dryness under reduced pressure. The residue was dissolved in 1 % neutral phosphate buffer and extracted with *n*-butanol. The butanol extract was concentrated *in vacuo*. Addition of ether precipitated 203 mg. of an almost white solid, which contained 80 units/mg.

Chemical properties of musarin

Musarin is an acid with an equivalent weight of *c.* 5000 as indicated by electrometric titration. The sodium or potassium salt is soluble in water, methanol, ethanol and butanol, but not in ether or acetone. The free acid is insoluble in water or acetone, but is soluble in 30 % aqueous acetone and alcohol. The acid is obtained as a flocculent precipitate when a neutral solution of musarin is acidified with dilute HCl, H_3PO_4 or H_2SO_4, but organic acids such as acetic or oxalic acid do not precipitate musarin completely. Musarin is also precipitated by $BaCl_2$, $HgCl_2$ or copper acetate, but the active material cannot be recovered from the precipitates or the supernatant liquid.

The free acid is somewhat unstable even when kept as the dry solid, but the sodium salt appears to be stable. The activity was unaltered at pH 2 or 11 at room temperature for 30 min. but was completely destroyed at 100° under these conditions. Heating at 100° at pH 7 caused 50 % inactivation after 30 min. and 60–80 % inactivation after 1 hr.

Table 2. *Chromatography of musarin on alumina*

Fraction	Vol. of eluate (ml.)	Wt. of solid (mg.)	Total units
1	35	20	80
2	30	143	3,600
3	20	335	27,000
4	35	270	21,500
5	110	70	4,500
6	80	Trace	—

Fraction 3: $[\alpha]_D^{24^\circ}=32\cdot3^\circ \pm 2\cdot4^\circ$. Recovery of active material from whole chromatogram = 33 %.

Musarin (80–100 units/mg.) could not be purified further by fractional precipitation with 0·0506 N-HCl or by chromatography on alumina. Chromatography was best carried out by using Merck's alumina which was adjusted to pH 5–6 with 50 % H_2SO_4 and washed thoroughly with 85 % aqueous methanol. In a typical experiment 2 g. of musarin $[\alpha]_D^{20^\circ}=35\cdot1^\circ \pm 1\cdot6^\circ$ (80 units/mg.) in 85 % aqueous methanol (50 ml.) were purified on 20 g. of Merck's alumina. The column was eluted with 85 % aqueous methanol. Each fraction was concentrated *in vacuo* to 5–10 ml. and the active material was precipitated with *c.* 100 ml. acetone (Table 2)

The most potent preparations of musarin sodium salt decomposed at about 170° without melting. The sodium salt was optically active $[\alpha]_D^{20^\circ}=35\cdot1^\circ \pm 1\cdot6^\circ$ (*c.* 1·21 in methanol); $[\alpha]_D^{20^\circ}=38\cdot7^\circ \pm 2\cdot7^\circ$ (*c.* 0·736 in methanol) and absorbed ultra-violet light: $\lambda_{\text{max.}}=2400$, 2670 (inflexion) A, $E^{1\%}_{1\,\text{cm.}}=375$, 200 (in ethanol). Analyses of the free acid (Found: C, 57·75; H, 8·34; N, 3·70 %) and of the sodium salt of similar potency (Found: C, 57·5, 56·8; H, 8·55, 8·27; N, 4·08, 4·25 %) were very similar, reflecting the high equivalent weight of musarin. Musarin did not contain sulphur, halogen or phosphorus. These data are in agreement with an empirical formula, $C_{35}H_{60}O_{14}N_2$ (requires C, 57·35; H, 8·2; N, 3·8 %).

Musarin gave negative Molisch, Millon and biuret tests for carbohydrate and protein respectively, but certain preparations gave a weak ninhydrin test after hydrolysis with 5 N-HCl at 100° for 2 min. Since this reaction was not given by all preparations it is not considered significant. Musarin gave no coloration with sulphuric acid in acetic acid or with iodine, and the Salkowski and Liebermann tests for steroids and cholesterol as well as the murexide test were also negative. Musarin did, however, give a positive Axenfeld reaction for protein, i.e. a red colour was obtained when a solution of gold chloride was added to a boiling solution previously acidified with formic acid; on addition of gold chloride a blue-violet colour developed.

Treatment of musarin with an ethereal solution of diazomethane resulted in an inactive product which was insoluble in 1 % neutral phosphate buffer. Similarly, treatment with methanol containing a few drops of methanolic HCl gave an inactive product, soluble in acetone and chloroform but insoluble in 1 % neutral phosphate buffer. This solid had an optical rotation $[\alpha]_D^{22^\circ} = 32 \cdot 2 \pm 2 \cdot 0^\circ$ (*c.* 0·995 in methanol).

Titration of musarin with acid and alkali. Micro-titration both of the free acid with sodium hydroxide and hydrochloric acid and of musarin sodium salt with hydrochloric acid was carried out in a special cell using a glass electrode (Catch, Cook & Kitchener, 1945). Titration of the free acid (40 units/mg.) indicated an equivalent weight of *c.* 8000, but of this musarin sodium salt (80 units/mg.), a value of *c.* 4000. The titration curves were complex, presumably due to the presence of other groups.

Biological properties of musarin

In all the following tests musarin containing 80 or 100 units/mg. was used.

Antifungal activity. Table 3 records the highest dilutions of musarin in which the germination of the spores of sixteen fungal species was completely inhibited. In most cases the next higher dilution delayed germination for 24–48 hr. and there was corresponding poor and slow mycelial development. In the lower dilutions most of the spores looked dead but no tests were made to prove this. The germination of spores of *Fusarium lini* was permanently inhibited only at 1/50,000 but in 1/100,000, although the spores germinated, they produced only short, hypertrophied germ-tubes which shrivelled and died. A dilution of musarin of 1/200,000 caused delay and poor development of mycelial growth in *F. lini*. It is apparent from Table 3 that musarin is a powerful antifungal agent, being especially active against the destructive plant parasites *Verticillium dahliae*, *V. albo-atrum*, *Botrytis cinerea* and *Ceratostomella paradoxa*.

Comparison of the antifungal activity of musarin with streptomycin and streptothricin. The antifungal activity of musarin was compared with that of a sample of streptomycin containing 700 units (Schatz, Bugie & Waksman, 1944) per mg. and of a sample of streptothricin containing 400 units (Foster & Woodruff, 1943) per mg., against the sixteen fungal species given in Table 3 (method *a*). Streptomycin was quite inactive against all the fungi and a concentration of 1/100 did not inhibit the germination of *Fusarium lateritium* spores in hanging-drop cultures.

Table 3. *Antifungal activity of musarin*
(80–100 *units/mg.*)

Fungus	Plant disease	Highest dilution affecting spore germination	
		Complete inhibition	Delay
Fusarium oxysporum var. *cubense*	Panama disease of banana	1/100,000	1/200,000 (48 hr.)
F. lateritium	Bud rot of fruit trees	1/100,000	1/200,000 (48 hr.)
F. culmorum	Foot rot of cereals, die-back of carnations	1/100,000	1/200,000 (48 hr.)
F. lini	Wilt of flax	1/50,000	1/200,000
Botrytis cinerea	Grey mould of lettuce	1/400,000	1/800,000*
Verticillium dahliae	Wilt of tomato and many other plants	1/800,000	1/1,600,000* (24 hr.)
V. albo-atrum		1/800,000	
Ceratostomella paradoxa	Pineapple disease of sugar cane	1/400,000 (No inhibition at 1/800,000)	
Rhizopus stolonifer	Soft rot of fruits	1/50,000	1/100,000 (24 hr.)
Corticum solani	Stem canker of potato, etc.	1/800,000	
Sclerotinia fructigena	Brown rot of apples, etc.	1/800,000	
Aspergillus parasiticus	—	1/50,000	1/100,000 (48 hr.)
A. niger		1/100,000 (No inhibition at 1/200,000)	
Penicillium notatum	—	1/50,000 (No inhibition at 1/100,000)	
Melanospora destruens	—	1/400,000	1/800,000
Alternaria sp.		1/400,000	

* Poor mycelial growth.

Streptothricin was active against all the fungi except *Aspergillus parasiticus*. It was about as potent as musarin against the four *Fusarium* strains, *Penicillium notatum* and *Aspergillus niger*, but was much less active against the other nine cultures; moreover, the inhibition of all growth in and over the surface of the clear zones was permanent on the musarin test plates but on the streptothricin plates the mycelial growth gradually spread all over the originally inhibited areas. For example, musarin produced a 15 mm. wide zone against

Ceratostomella paradoxa and this area was still entirely free from growth 25 days later; streptothricin produced a 10 mm. wide zone which was reduced in 3 days to 3 mm. by the growth of mycelium over the surface.

Antibacterial activity of musarin. Musarin, in a concentration of 1/500, produced a 7 mm. wide inhibition zone in plates of *Staph. aureus.* From these tests and from similar tests against *B. subtilis* and *Strep. pyogenes* it appeared that musarin was only feebly active against Gram-positive bacteria; but when broth dilutions were made it was found that musarin was very active against *Staph. aureus*, completely inhibiting growth at 1/500,000. The highest effective dilutions of musarin against five bacterial species is shown in Table 4.

Table 4. *Antibacterial activity of musarin* (80–100 *units/mg.*)

Bacterium	Highest effective dilution
Staph. aureus	1/500,000
B. subtilis	1/100,000
Strep. pyogenes	1/25,000
Myco. phlei I	1/100,000
Bact. coli	Less than 1/5000

It was evident that the agar-plate method gave no criterion of the antibacterial activity of musarin, although it gave a fairly accurate measure of antifungal activity. Two explanations of the divergence of results seemed possible: musarin might diffuse into an acid but not into an alkaline medium, for whereas the fungal tests were made on acid malt-agar, the bacterial tests were made on medium of pH 7·2; or musarin might diffuse so slowly into the medium that the rapidly growing *Staph. aureus* colonies were established before the antibiotic had reached beyond the 7 mm. zone. Owing to the slower rate of growth of fungi, the fungal plates would not be so affected by this factor. Experiments showed that the second explanation was correct. Thus, a 15 mm. wide zone developed round a 1/1000 solution of musarin on a test plate of *Staph. aureus* held at room temperature, whereas a control plate, incubated at 37° produced the usual 7 mm. zone only.

Comparison of the antibacterial activity of musarin, streptomycin and streptothricin. Broth dilution tests showed that streptomycin and streptothricin were much more active than musarin against *Myco. phlei.* The limiting titre of musarin was 1/100,000, but streptomycin (700 units/mg.) and streptothricin (400 units/mg.) completely inhibited the growth of *Myco. phlei* at 1/250,000, the highest dilution tested. Musarin was more active against *Staph. aureus* than either streptomycin or streptothricin. All three antibiotics were about equally active against *Strep. pyogenes* but streptomycin was less active against *B. subtilis* than either musarin or streptothricin. The greatest difference, however, in antibacterial activity was against *Bact. coli*, for whereas both streptomycin and streptothricin were effective against this organism in about 1/75,000 dilution, there was heavy growth of *Bact. coli* even in the 1/5000 dilutions of musarin (Table 5).

Table 5. *Antibiotic activity of musarin* (80–100 *units/mg.*), *streptomycin* (700 *Waksman units/mg.*) *and streptothricin* (400 *Foster & Woodruff units/mg.*)

		Highest effective dilution of	
Test organism	Musarin	Streptomycin	Streptothricin
General antifungal activity	Very strong, permanent	None	Strong, transient
Aspergillus parasiticus	1/50,000	None	None
Verticillium sp.	1/800,000	None	1/250,000
Rhizoctonia sp.	1/800,000	None	1/50,000
Ceratostomella sp.	1/400,000	None	1/50,000
Staph. aureus	1/500,000	1/100,000	1/100,000
Myco. phlei I	1/100,000	<1/250,000	<1/250,000
Bact. coli	>1/5000	1/75,000	1/75,000

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An Apparatus made from Meccano Parts for the Preparation of Roll-tube Cultures

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SUMMARY: Equipment can be readily made in the laboratory for the mechanical preparation of roll-tubes. Roll-tubes so prepared can largely replace Petri dishes both for counting and for single colony isolations.

The recent shortage of Petri dishes has compelled the adoption of an alternative method for counting viable bacteria. The roll-tube method, which consists essentially in the replacement of Petri dishes by test-tubes, was chosen (Wilson, 1922). Apart from considerations of accuracy, rolled tubes are more economical than Petri dishes since ordinary test-tubes may be used; only one-fifth of the medium is required and the number of dilutions is reduced since it is customary to use a standardized dropping pipette to deliver a known volume of inoculum into the tube. Against this must be set the disadvantage that streaking and picking of individual colonies requires a greater manipulative skill than with Petri dishes.

Withell (1938) obtained a standard error of ±5% with roll-tube counts. Using similar statistical methods Jenninson & Wadsworth (1940) obtained a similar figure for the plate method; with a reasonable number of replicates and only three dilutions the standard error was ±6·6%.

Uniform roll-tubes are difficult to prepare without mechanical aid. Thompson (1934) described an apparatus for making them, and a successful commercial model was apparently obtainable in America before the recent war (Prouty, Bendixen & Swenson, 1944). Based on published photographs of this model, an apparatus was constructed from Meccano parts. It is simple in design, ordinary test-tubes (1·5 × 25 cm.) with cotton-wool plugs are used, and it has proved successful in daily use for 3 months.

THE APPARATUS

Test-tubes containing 2 ml. of melted-agar (2%) medium are inoculated and placed in the machine as shown in Pl. 1, fig. 1. The whole machine is inclined from front to back at an angle of 9° so that the melted agar remains in the bottom 4 cm. of the tubes. The tubes are then rotated. The agar creeps up to within 2 cm. of the cotton-wool plug and sets in about 2 min. Friction between the hinged front plate *D* and the cotton-wool plug reduces the speed of rotation from 950 r.p.m. (unloaded apparatus) to 650 r.p.m. when fully loaded with the four tubes.

The general arrangement of the equipment may be seen in Pl. 1, figs. 1 and 2. The rollers which cause the tubes to rotate and the main shaft are carried on a substantial framework. The frame itself is simple and consists of right-angled girders made rigid with base plates and cross-girders. The apparatus measures 2 ft. × 5½ in. at the base and is 12 in. high.

The bearings. Each tube rests on four wheels of which the two pairs of driving wheels F (Pl. 1, fig. 2) are fixed on their spindles, while the others are free running. Each pair of driving wheels, which have rubber tyres (a strip of bicycle inner-tubing) to ensure adequate friction, rotates two tubes in an anticlockwise direction. The idler wheels on arm G are set at such an angle to the axes of rotation of the driving wheels that the tubes tend to thrust against the back plate E, instead of the hinged front plate D. Friction against the front plate D must be minimized to avoid undue loss of speed and to prevent formation of large amounts of cotton-wool dust. Some cotton-wool dust is inevitable and for this reason the rubber-band drive is preferable to a geared drive.

Method of siting the tubes

Each tube is supported on four wheels and enclosed front and back by metal plates. If the apparatus is perfectly adjusted the hinged front plate D becomes unnecessary, but this condition is difficult to maintain in practice.

The tubes are held in contact with the rollers by means of the spring-loaded idler wheels H_1–H_4, carried on the hinged arm G which is loaded with a 200 g. weight (see Pl. 1, figs. 1 and 2). These idler wheels are Meccano pulleys no. 23 fitted with rubber tyres. They are under tension by the action of small compression springs I_1–I_4, and held in position with a collar K. The fixing screw L of collar K is sufficiently long to enable the screw and collar to slip behind another screw M. This arrangement prevents lateral movement of the idler wheels but allows free movement in the vertical plane. Contrary to expectation, it was found in practice that best results were obtained if the tension spring I_1 was stronger than the other tension springs.

The arm G, which is mounted on a plate N, has a sliding adjustment so as to allow the idler wheels H to be brought centrally over each tube.

The drive. The drive is clearly shown in Pl. 1, fig. 2. Pulley wheel A transmits the power from a 1/5 h.p. motor to pulleys B and C. The drive to the spindle of the rollers is effected by means of rubber bands attached through a right-angle turn.

The counter. The colonies are counted in the apparatus shown in Pl. 1, fig. 3, which is a slowly rotating support for the tubes set against a black background.

Streak tubes can readily be prepared by means of this equipment. The bottom of a roll-tube containing the sterile medium is gripped in the clip, the tube is rotated (at a greater speed than for counting) and after removing the plug the charged loop is inserted and slowly withdrawn in contact with the agar when a spiral streak will result.

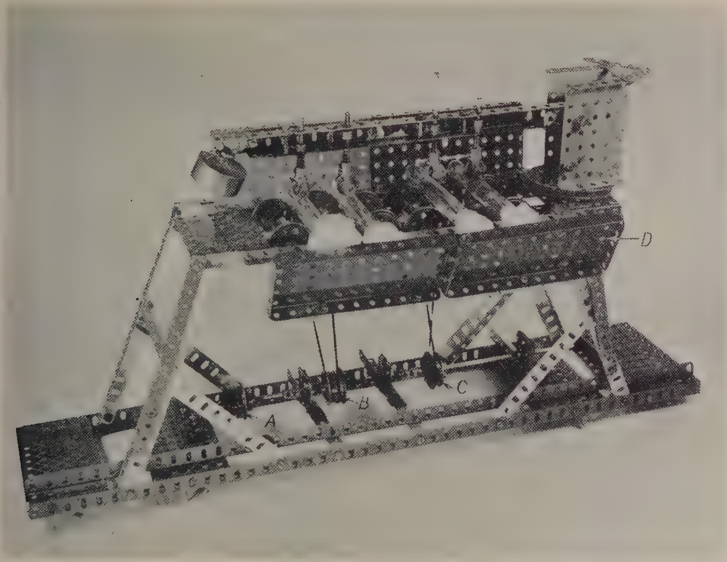
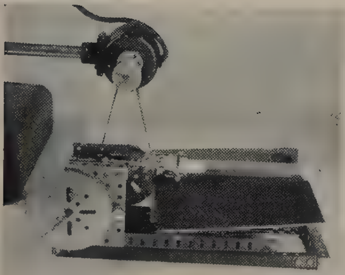
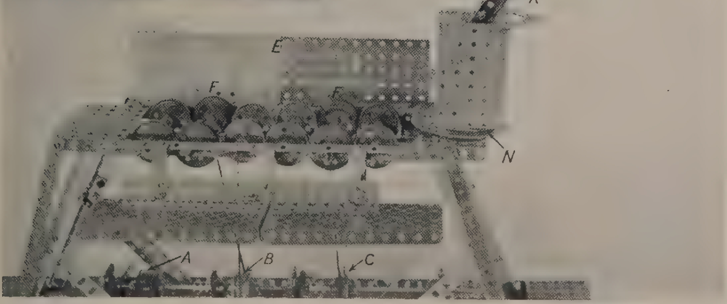


Fig. 1

Fig. 3




G
H_1
L
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H_2
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H_3
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I_4
M
K
E
F
F
N
A
B
C

Fig. 2

A. Hirsch—An apparatus made from Meccano parts for the preparation of roll-tube cultures. Plate 1

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EXPLANATION OF PLATE

Fig. 1. The apparatus with four roll-tubes in position.
Fig. 2. The apparatus with the arm bearing the idler wheels raised.
Fig. 3. Slowly rotating holder for counting roll-tube cultures.

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The Cytology of the Gram-positive Cocci

By K. A. BISSET

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SUMMARY: The long-chained and short-chained variants of *Streptococcus pyogenes*, *Str. viridans* and *Str. faecalis* possess a structure closely resembling that of rough and smooth bacillary variants. Cocci of the long-chained strains are frequently divided by transverse cell walls into two cells, each containing a single chromatinic body. Short-chained strains fail to form transverse cell walls, the cocci dividing by constriction; they are unicellular and usually contain a pair of chromatinic bodies. Analogous types occur in *Staphylococcus aureus* and *Staph. albus*. In one of these types the cells contained a well-defined central granule, dividing with the coccus.

It has been pointed out (Bisset, 1938) that there is a close analogy between the smooth and rough morphological types of rod-shaped bacteria on the one hand, and the short-chained and long-chained streptococci and pneumococci on the other. The long-chained streptococci, like the rough bacilli, adhere to one another after cell division and form a 'Medusa-head' colony (Pl. 1, fig. 1) whereas the short-chained cocci, like the smooth bacilli, separate more or less completely, and form relatively structureless colonies (Pl. 1, fig. 2). This variation is entirely independent of those usually described as S → R in the pneumococcus, or 'matt' → 'glossy' in the streptococcus, each of which is concerned only with the capsular material. In a recent paper (Bisset, 1947) the author has shown that rough variants of species both in the *Bacteriaceae* and the *Bacillaceae* are often divided by transverse cell walls and septa into several cells, each containing a single chromatinic body. The unicellular smooth variants contain a pair of bodies and do not form transverse cell walls. In the present study the minute structure of the two types of streptococcus, and of certain types of staphylococcus, is compared with that of the bacillary variants.

Nuclear structures have been reported in various Gram-positive cocci (Piekarski, 1938; Robinow, 1942; Knaysi, 1942), and single, double, and multiple nuclei have been described, although not in a streptococcus. Knaysi & Mudd (1943) failed to demonstrate nuclear structures in a streptococcus with the electron microscope, but claimed to have done so in a staphylococcus.

METHODS

The osmic-acid, HCl-Giemsa, and tannic-acid-violet techniques of Robinow (1945) were used throughout, and water mounts were always employed. The value of these techniques in the examination of bacterial chromatinic apparatus and cell walls depends upon the removal of the staining capacity of those portions of the cell which it is not intended to demonstrate. By hydrolysis with HCl, the polysaccharide material which probably forms the major portion of the cell wall is rendered unstainable and at least partially destroyed. After this treatment bacteria appear much reduced in size by the loss of this surface material. Tannic acid leaves the cell wall unharmed, and destroys or renders unstainable the protein contents of the cell, which appears as if empty. Taken together, these methods probably give a reasonable picture of the structure of bacteria.

The streptococci studied were stock cultures of *Streptococcus pyogenes*, *Str. viridans* and *Str. faecalis* used for the production of Lancefield grouping sera, and strains recently isolated from both the human subject and dogs. They were of various antigenic groups and were either alpha- or beta-haemolytic. Neither of these characters was correlated with the morphological variation under examination; the strains were either long-chained or short-chained upon isolation and some had since produced variants. Colony form and habit of chain length was examined by means of impression preparations (Bisset, 1938).

Strains of *Staphylococcus aureus* and *Staph. albus* were examined. Some strains were stock cultures, and others were newly isolated from pathological material or from random, air-borne contaminants. A stock culture of *Sarcina lutea* was also examined. Cultures were made upon horse-blood agar plates, and were examined when about 24 hr. old.

OBSERVATIONS

When stained by tannic-acid-violet to demonstrate the cell walls, the two types of streptococcus were markedly different in appearance. The long-chained forms, which were mainly *Streptococcus pyogenes* and *Str. viridans*, were almost completely spherical, and a large proportion of cocci had obvious transverse septa, continuous with the cell wall. Recently divided cocci were closely applied together, and no great degree of constriction was observed in their division (Pl. 1, fig. 3). The short-chained forms, which were strains of *Str. pyogenes* and *Str. faecalis*, were lanceolate in outline and without transverse septa. Marked constrictions appeared between the dividing cocci and separation was complete or almost so (Pl. 1, fig. 4). The newly formed cell walls between recently divided cells of this type were unmistakably different from the septa, which, in the long-chained forms, halved otherwise entire cocci. The occasional long filaments which appeared in both types of coccus, as also in both S and R bacilli, occasionally exhibited what appeared to be transverse septa, even in the short-chained types. As the same filament might also show signs of division by constriction, however, it is probable that these apparent septa were indicative of past, rather than future division, and merely marked the failure of the new sub-units to separate after the formation of the new cell wall (Pl. 1, fig. 5).

When stained with Giemsa, after hydrolysis for about 2 hr., long-chained cocci showed a varied appearance (Pl. 1, fig. 6). In the majority of cells the protoplasm retained its staining capacity, the most obvious feature being the unstained bar, representing the septum. Where hydrolysis was more complete, the only structures retaining the stain were relatively long, narrow chromatinic bodies, arranged in pairs. These pairs were sometimes so close together as to be almost touching, and sometimes more widely separated.

By superimposing tracings of photographs, taken at the same magnification, it was possible to establish that the former pairs were capable of being contained within a single coccus, presumably on the point of division, whereas the latter were so spaced as to lie in each half of a divided or septate organism (Fig. 1). In the short-chained form stained by acid-Giemsa, the majority of cocci contained two bodies, which, though apparently spherical, were probably merely unresolved. A minority contained only one (Pl. 2, fig. 7; Fig. 2).

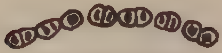

Fig. 1. Composite tracing from Pl. 1, figs. 3 and 6.

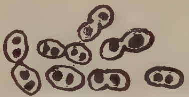

Fig. 2. Composite tracing from Pl. 1, fig. 4 and Pl. 2, fig. 7.

The majority of strains of both *Staph. aureus* and *Staph. albus* examined bore a close resemblance, in their minute structure, to long-chained streptococci. Well-marked transverse septa were observed (Pl. 2, fig. 8) their position being indicated in the Giemsa preparations by an unstained bar across the middle of the coccus (Pl. 2, fig. 9). Unlike the streptococci, however, the succeeding division was often at right angles to the preceding one, and it was common to find a recently divided coccus in which one of the daughter cells had subdivided in this manner, before complete separation had occurred (Pl. 2, *a* in figs. 8 and 9). Cells of abnormal length were not found, but occasional cocci, greatly swollen in all dimensions, were to be seen. In the ordinary processes of growth the increase in size was three-dimensional, and not by elongation as in the streptococci. In staphylococci with this morphology a unicellular coccus was the exception rather than the rule. Chromatinic bodies were even less clearly defined than in the long-chained streptococci, appearing merely as comparatively heavily stained central areas in the cytoplasm. The cytoplasm strongly resisted hydrolysis with HCl and retained its staining capacity after many hours. The second morphological type of staphylococcus was much less commonly found, although it included saprophytic *albus* strains and haemolytic *aureus* strains isolated from pathological material. The cells contained a clearly defined granule, central or slightly eccentric in position (Pl. 2, fig. 10). Division of the granule preceded that of the coccus, and growth was mainly, although not exclusively, by elongation. This type of coccus did not form transverse septa, and was apparently unicellular (Pl. 2, fig. 11). Morphologically this type of staphylococcus was similar to the strain of *Sarcina lutea* examined, although only the *Sarcina* was arranged in the typical packet-of-eight. The existence of discrete, central chromatinic bodies in this genus is already fully recorded (Piekarski, 1938; Robinow, 1942).

DISCUSSION

The demonstration of chromatinic bodies in *Streptococcus* and *Staphylococcus* by means of Robinow's modification of Feulgen's reaction, serves merely to bring these genera into line with the other bacterial groups which have been studied in this way. A separate discussion of the significance of the finding is not appropriate to this paper. The present observations are of assistance, however, in the interpretation of some of the previously recorded observations upon the nuclear apparatus of staphylococci. Knaysi (1942) appears, from his photographs, to have been observing a septate staphylococcus, although the septa are not described. The multiple granules demonstrated in a staphylococcus by Knaysi & Mudd (1943), by means of the electron microscope, may possibly be interpreted as the effect of desiccation upon the same type of coccus in a multicellular condition, as in Fig. 5, no. 2.

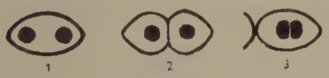

Fig. 3

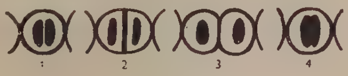

Fig. 4

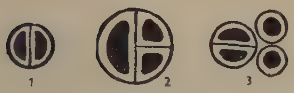

Fig. 5

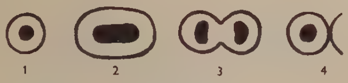

Fig. 6

Fig. 3. Short-chained streptococcus in process of division. Suggested interpretation.
Fig. 4. Long-chained streptococcus in process of division. Suggested interpretation.
Fig. 5. Septate staphylococcus in process of division. Suggested interpretation.
Fig. 6. Non-septate staphylococcus in process of division. Suggested interpretation.

It is of interest to observe that the parallel between smooth and rough variants in the bacillary genera, and short- and long-chained streptococci, appears to extend to their minute structure. In both groups, the variant which adheres in chains after division, and consequently forms 'Medusa-head' colonies, also produces multicellular bacilli or cocci, possessing transverse cell walls, and with each cell containing a single chromatinic body, except, probably, immediately before division (Fig. 4). Similarly, in both groups, the variant which separates completely after division fails to form transverse cell walls, and divides by constriction, producing unicellular bacteria, each of which contains a pair of chromatinic bodies (Fig. 3). This resemblance between the streptococci and the bacillary genera is enhanced by the observation that both types of streptococcus grow by elongation, and when normal cell division fails to take place produce long, filamentous elements of normal breadth, analogous to those occurring in cultures of bacilli. In fact, the only basic difference between a streptococcus and a bacillus is average cell length.

Although the staphylococci also produced septate and non-septate forms, the analogy cannot at present be carried any further. The septate form does indeed resemble closely the long-chained streptococcus, but differs in that it is a true, radially symmetrical coccus, increasing in size in all dimensions, and capable of dividing in any plane (Fig. 5). The non-septate form is quite unlike either of the streptococcal forms in its possession of a single chromatinic granule, which, from its resemblance to the acknowledged nuclear apparatus of *Sarcina*, can fairly be regarded as a discrete nucleus (Fig. 6).

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Long-chained streptococcus colony, impression preparation. × 500.

Fig. 2. Short-chained streptococcus colony, impression preparation. × 500.

Fig. 3. Long-chained streptococcus showing transverse septa. Tannic-acid-violet. × 3000.

Fig. 4. Short-chained streptococcus dividing by constriction. Tannic-acid-violet. × 3000.

Fig. 5. Short-chained streptococcus. The long filament shows a point of constriction near one end, and an apparent septum, probably representing an uncompleted division. Tannic-acid-violet. × 3000.

Fig. 6. Long-chained streptococcus. The central group is fully hydrolysed and only the pairs of chromatinic bodies are stained. In the rest of the field the entire cytoplasm of the cell is stained in most cases, and the unstained gap, representing the septum, is clearly shown. Acid-Giemsa. × 3000.

Plate 2

Fig. 7. Short-chained streptococcus showing double chromatinic bodies in most of the cells. Acid-Giemsa. × 3000.

Fig. 8. Septate staphylococcus. *a* is a coccus dividing at right angles to the previous division. Tannic-acid-violet. × 3000.

Fig. 9. Septate staphylococcus. *a* is a coccus producing a secondary septum at right angles to the primary one. Acid-Giemsa. × 3000.

Fig. 10. Non-septate staphylococcus. Acid-Giemsa. × 3000.

Fig. 11. Non-septate staphylococcus. Tannic-acid-violet. × 3000.

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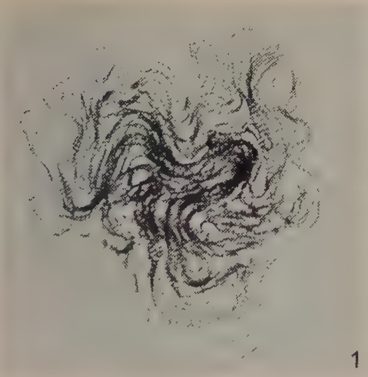
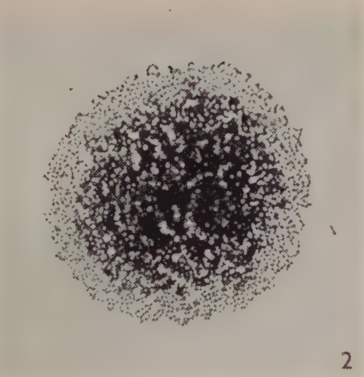
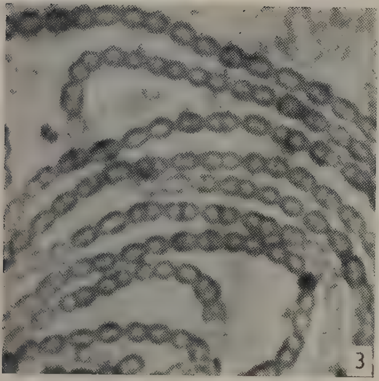
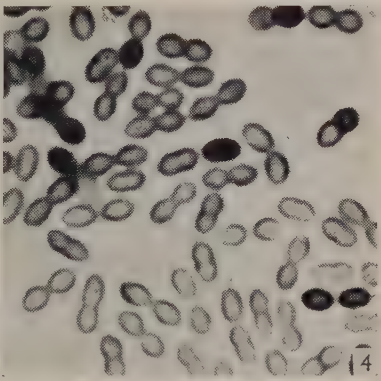
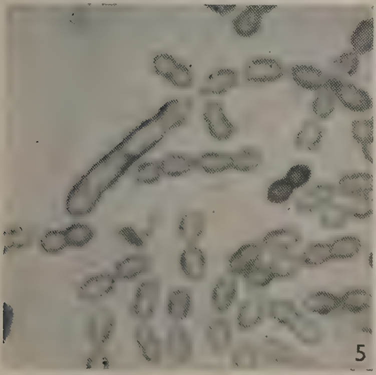
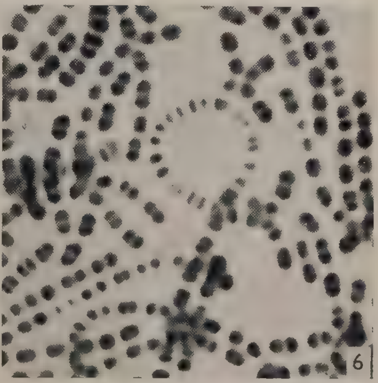






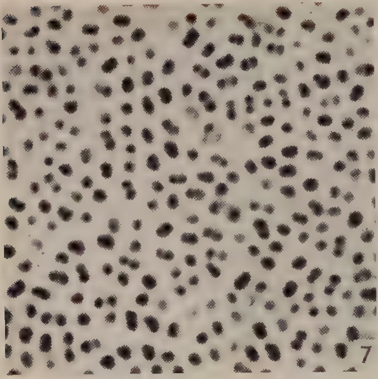


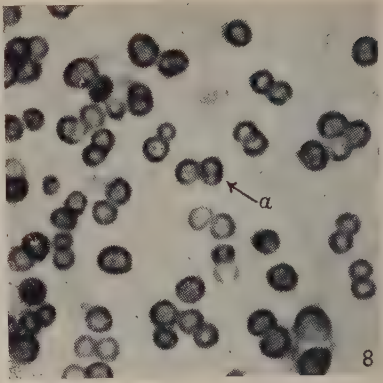


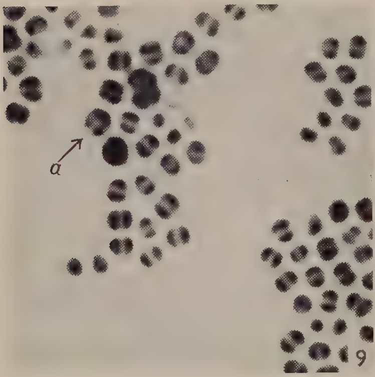


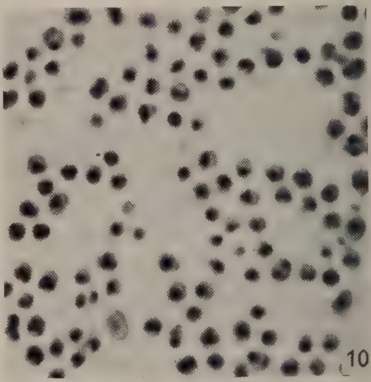
Figs. 1–6

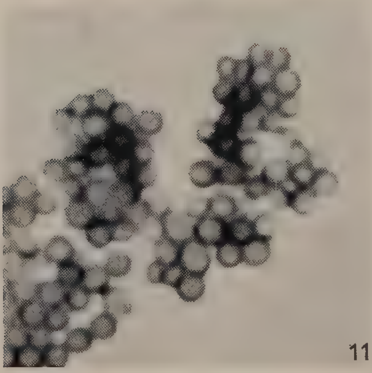
K. A. BISSET—THE CYTOLOGY OF THE GRAM-POSITIVE COCCI. PLATE 1











Figs. 7–11

K. A. Bisset—The cytology of the gram-positive cocci. Plate 2

A Method for the Measurement of Mutation Rate from Phage Sensitivity to Phage Resistance in *Escherichia coli*

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SUMMARY: The growth of *Escherichia coli* strain *B/r* on nutrient agar is the same as in broth with regard to the initial lag period and the rate of increase during the exponential phase. On nutrient agar, growth is finally slowed down either when individual colonies contain more than 2^{19} bacteria, or when the total number of bacteria/4 in. plate exceeds 10^{10}.

The rate of mutation to phage resistance was measured by a modification of Demerec's method of spraying plate cultures with a phage aerosol, and counting resistant survivals. With the phage *T*1, after twelve bacterial generations on nutrient agar at 37°, the value of $0{\cdot}74 \pm 0{\cdot}04$ mutations/10^8 bacterial divisions was obtained, and after eighteen bacterial generations, $0{\cdot}70 \pm 0{\cdot}04$.

Resistance to *T*1 phage is probably brought about by at least five separate mutations, as shown by tests of resistance to other phages. Different strains of *E. coli* yield different proportions of some of these mutants. Thus strain *B/r* yielded a lower proportion of the mutant *B*/1 than did strain *B*. Furthermore, as different strains of *T*1 phage may contain varying amounts of the phage mutant *T*1*h*, capable of lysing the bacterial mutant *B*/1, but not *B*/1, 5, variations in the relative proportions of *B*/1 and *B*/1, 5 may also occur when different samples of phage are used. Consequently, constancy of mutation rate can only be guaranteed for given cultures of both bacteria and phage.

A method for the measurement of the rate at which phage-resistant cells of *E. coli* arise in phage-sensitive cultures has been described by Luria & Delbrück (1943), who adopted the technique of growing cultures of the bacterium for given periods of time in liquid broth media, and subsequently plating out known small amounts from the cultures on to nutrient agar plates, together with an excess of phage particles. All except resistant bacteria were then killed, and the proportion of the latter could easily be determined by allowing them to grow into visible colonies. The calculation of the rate at which sensitive bacteria gave rise to resistant ones was however complicated by the fact that the proportion of resistant bacteria depended largely on the time at which mutation took place, because a single early mutation would lead to the formation of a large clone of resistant bacteria, and a late one would yield only one or two resistant cells. Luria & Delbrück attempted to overcome this difficulty (1) by basing their calculations on the proportion of cultures which did not contain any resistant mutants at all, and (2) by utilizing the average number of resistant bacteria per culture. The first method, which makes use of only a part of the available information, gave a mutation rate of $0{\cdot}32 \times 10^{-8}$/bacterium/division cycle, and the second method, which is inaccurate on account of the assumption that there are no early mutations, gave values varying between 0·76 and $2{\cdot}8 \times 10^{-8}$.

Demerec (1946) modified the above method by growing the bacteria on nutrient agar instead of in a liquid medium, and by applying the phage as a fine aerosol sprayed on to the plates. When grown on concentrated agar the bacteria do not move about to any serious extent, and consequently all mutations occurring after plating are represented by single colonies, no matter how many bacteria have been formed by multiplication of the original mutant cell. The difficulty is now to determine precisely the number of bacteria on a plate at the moment of spraying with phage. It is the object of the work here described to show how this may be done and, from the data obtained, to make a more precise determination of the mutation rate from phage sensitivity to phage resistance. The advantage of this method is that it enables one to determine the mutation rate at a given time in the development of a bacterial colony, while the method of Luria & Delbrück gave only an average for the rates over the whole period of growth.

METHODS

General

The cultures of *E. coli* used in all experiments here described were made from the radiation resistant *B/r* strain of Witkin (1946), with the exception of the tests described in the section on *Sub-classification of mutants* (p. 139), where strain *B* (from which *B/r* was originally derived) was also used. Phage cultures were prepared from the *T*1 strain. Growth and mutation rates were studied on standard media prepared from Difco nutrient agar, 2·3 g./l., together with NaCl, 5 g./l., and a trace of gentian violet to diminish the chance of contamination by air-borne Gram-positive bacteria. All cultures were incubated at 37°. Care was taken to ensure that the Petri dishes were kept at this temperature right up to the moment of plating the bacteria, and to minimize the time during which the plates were exposed to room temperature—(which was never more than 2 min.). Growth was checked at any desired time by transferring the plates to the cooling element of a refrigerator ($-5°$) for 3 min., and afterwards storing them in the main chamber ($+5°$) until required. At 5° neither growth nor death occurs appreciably during a few hours.

Spraying of phage was carried out as described by Demerec (1946), except that a larger type of nebulizer (no. 40) was found to be necessary in order to deliver enough phage particles on to the surface of the agar in 2 min. After spraying with phage, cultures were incubated for at least 48 hr. to permit resistant colonies to become easily visible. With a shorter period of incubation, the slower-growing colonies were sometimes missed.

Growth rate of Escherichia coli *on nutrient agar*

The number of living bacteria on a 4 in. agar plate was estimated by pipetting on to the surface successive samples each of 5 ml. 0·85 % saline. After each washing, the bacteria were scraped off the agar with a glass spreader, and the suspensions thus obtained pipetted off and counted.

The effectiveness of this washing process was tested by pipetting on to a series of plates a suspension containing a known number of bacteria, spreading the drop, allowing to dry, and finally washing the bacteria off again before there was time for multiplication to have taken place. Table 1*a* shows the relative amounts of bacteria recovered in each of five successive washings of the same plate. On the average, 81 % of the total recovered were recovered in the first washing. In Table 1*b* the number of bacteria recovered in the first washing is compared with the number put on, and the mean here is 84 %. Thus the washing process is effective, and four successive washings would remove almost all the bacteria from a plate. This method was used for determining the growth rate of bacteria on nutrient agar, as described below. Subsequently, however, the technique was simplified by making a single washing with 10 ml. saline, stirring up thoroughly, and counting a 0·1 ml. sample pipetted directly from the flooded plate, instead of pooling the entire washings into a separate tube and counting the pool. The simplified method was found to be just as reliable as the first, and much quicker.

Table 1*a*. *Percentage distribution of bacteria in five successive 5 ml. saline washings of bacterial plate cultures*

	Plate								
Washing	1	2	3	4	5	6	7	8	Mean
1st	87·8	71·0	73·8	64·9	85·3	87·3	91·8	88·6	81·3
2nd	11·3	23·0	21·2	25·3	12·3	11·1	6·9	9·6	15·1
3rd	0·7	4·3	4·0	6·1	1·7	1·5	1·1	1·4	2·6
4th	0·3	1·0	0·8	3·4	0·4	0·4	0·1	0·4	0·8
5th	0·01	0·2	0·1	0·3	0·08	0·25	0·03	0·08	0·1

Table 1*b*. *Comparison of number of bacteria plated with number recovered in first washing of the plate culture*

	Plate					
	1	2	3	4	5	Mean
No. plated ($\times 10^6$)	197	182	204	209	234	205
No. recovered in 1st washing ($\times 10^6$)	134	206	168	183	184	174; or 84 %

Using the method of four successive washings, each of 5 ml. saline, pouring off and counting a mixture of all four washings, the rate of increase of *E. coli* (strain *B/r*) on nutrient agar at 37° was determined for periods ranging from 0 to 12 hr. (Table 2 and Fig. 1). A fully grown, aerated broth culture was used as source of bacteria in these experiments, which were made over a period of 6 days. It will be seen from the graph that there is an initial lag period of about 1½ hr. Growth then proceeds exponentially for about 8 hr., when the rate of increase diminishes. The slope of the curve indicates a division cycle of about 20 min. Thus the growth rate of *E. coli* (strain *B/r*) is essentially the same as that determined in broth by Witkin (1946). Similar results have recently been reported for another strain of *E. coli* by Mayr-Harting (1947), using somewhat different methods.

Table 2. *Growth rate of* E. coli *(B/r) on nutrient agar, measured by the washing method*

Growth at 37° (hr.)	Multiplication of number of bacteria plated. Plate 1	Plate 2	Plate 3	Plate 4	Mean
1½	1·8	1·2	1·1	1·2	1·2
2	2·6	—	—	2·1	2·3
2½	7·2	5·6	$1{\cdot}7 \times 10$	8·5	9·6
3½	$6{\cdot}3 \times 10$	$4{\cdot}6 \times 10$	$5{\cdot}6 \times 10$	$9{\cdot}6 \times 10$	$6{\cdot}5 \times 10$
4	$1{\cdot}7 \times 10^{2}$	—	—	—	$1{\cdot}7 \times 10^{2}$
4½	$3{\cdot}5 \times 10^{2}$	$4{\cdot}5 \times 10^{2}$	$3{\cdot}6 \times 10^{2}$	$3{\cdot}4 \times 10^{2}$	$3{\cdot}7 \times 10^{2}$
5	$1{\cdot}6 \times 10^{3}$	$2{\cdot}6 \times 10^{3}$	—	—	$2{\cdot}1 \times 10^{3}$
5½	$3{\cdot}7 \times 10^{3}$	—	$5{\cdot}9 \times 10^{3}$	$2{\cdot}8 \times 10^{3}$	$4{\cdot}2 \times 10^{3}$
6½	—	$3{\cdot}3 \times 10^{4}$	—	$2{\cdot}4 \times 10^{4}$	$2{\cdot}8 \times 10^{4}$
7	—	$7{\cdot}6 \times 10^{4}$	$9{\cdot}2 \times 10^{4}$	—	$8{\cdot}4 \times 10^{4}$
7½	—	$2{\cdot}6 \times 10^{5}$	$2{\cdot}8 \times 10^{5}$	$2{\cdot}5 \times 10^{5}$	$2{\cdot}6 \times 10^{5}$
8	—	—	—	$2{\cdot}9 \times 10^{5}$	$2{\cdot}9 \times 10^{5}$
8½	—	—	$1{\cdot}8 \times 10^{6}$	—	$1{\cdot}8 \times 10^{6}$
9	—	$2{\cdot}9 \times 10^{6}$	$1{\cdot}9 \times 10^{6}$	$3{\cdot}0 \times 10^{6}$	$2{\cdot}6 \times 10^{6}$
9½	—	$5{\cdot}3 \times 10^{6}$	$3{\cdot}2 \times 10^{6}$	$6{\cdot}6 \times 10^{6}$	$5{\cdot}0 \times 10^{6}$
10	—	$4{\cdot}9 \times 10^{6}$	—	$6{\cdot}0 \times 10^{6}$	$5{\cdot}4 \times 10^{6}$
10½	—	$7{\cdot}4 \times 10^{6}$	$1{\cdot}4 \times 10^{7}$	$8{\cdot}0 \times 10^{6}$	$9{\cdot}8 \times 10^{6}$
11	—	—	$1{\cdot}2 \times 10^{7}$	$9{\cdot}0 \times 10^{6}$	$1{\cdot}0 \times 10^{7}$
11½	—	—	$1{\cdot}9 \times 10^{7}$	$1{\cdot}2 \times 10^{7}$	$1{\cdot}5 \times 10^{7}$
12	—	—	$2{\cdot}1 \times 10^{7}$	$1{\cdot}2 \times 10^{7}$	$1{\cdot}6 \times 10^{7}$

Initial lag period on nutrient agar and in liquid broth media

In order to determine the initial lag period with bacteria growing on nutrient agar and compare it with the lag of similar samples of bacteria growing in liquid broth media, three fully grown cultures of strain *B/r* of different ages were taken, and the lag determined on agar and in broth for each. The results (Table 3) indicate that the lag periods are the same in the two media.

Table 3. *Initial lag period of* E. coli *(B/r) grown on nutrient agar and in broth*

Age of culture (days)	Lag period (min.) In broth	On agar
2	78	70
8	96	92
20	114	120

Effect of crowding on growth rate on nutrient agar

The effect of high densities of bacteria on the growth rate on nutrient agar is shown in Table 4. Series of platings were made with varying numbers of bacteria and the plates incubated for 5½, 6½ or 7 hr. The initial number of bacteria was so chosen as to lead to the production of between 10^9 and 10^{11} bacteria/plate after incubation. It was found that when the final number of bacteria is greater than 10^{10}, growth rate was markedly diminished (Table 4, cols. 3 and 4).

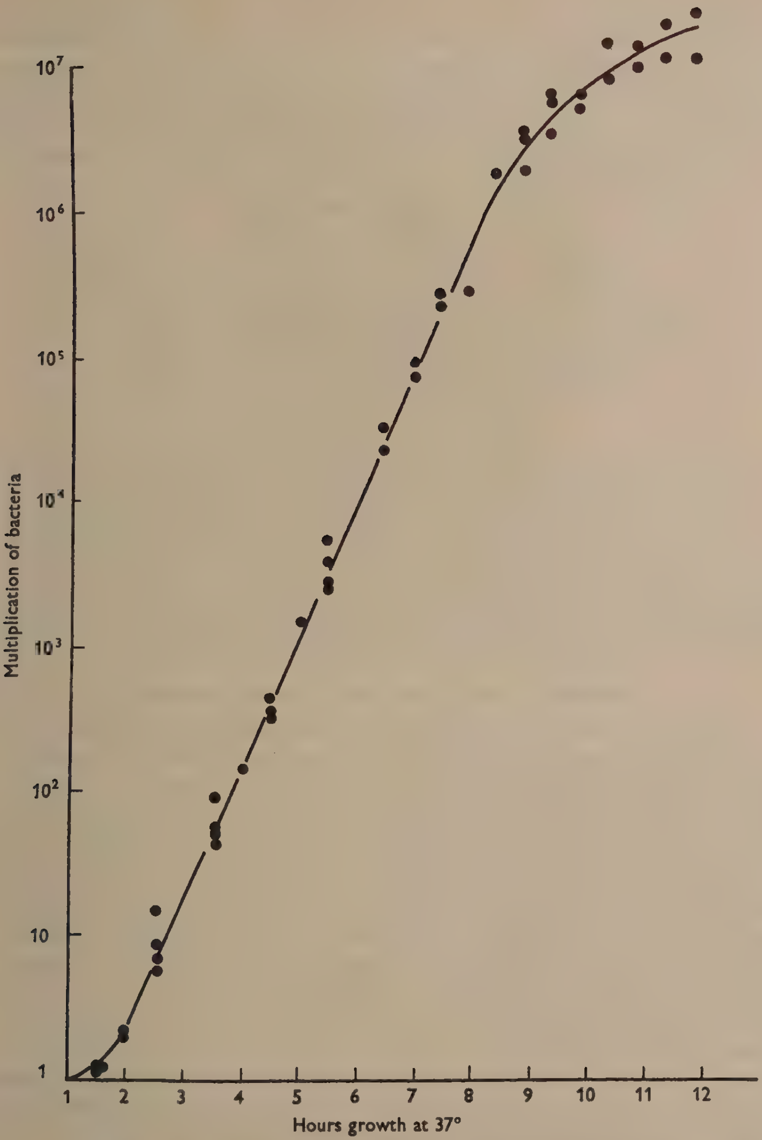

Fig. 1. Growth rate of *E. coli* (strain *B/r*) on nutrient agar at 37°. Data from Table 2.

It has already been shown (see Table 2 and Fig. 1) that growth rate of bacteria in individual colonies slows down after 8 hr., or about nineteen generations, no matter how small the number of colonies/plate. Now we have a second limitation of the exponential growth period, namely the total number of bacteria/plate, which must not exceed 10^{10}. Consequently, there may be a restriction of growth either within colonies, when they contain more than 2^{19} cells, or between colonies, when the total bacteria/plate is more than 10^{10}

Table 4. *Effect of crowding on growth rate of* E. coli (*B/r*) *on nutrient agar*

Growth at 37° (hr.)	No. of bacteria plated	Final no. of bacteria	Theoretical final no. assuming exponential growth
5½	2×10^8	5×10^{10}	8×10^{11}
	4×10^6	9×10^9	2×10^{10}
	2×10^6	7×10^9	8×10^9
	1×10^6	$2{\cdot}5 \times 10^9$	4×10^9
	4×10^5	2×10^9	2×10^9
6½	2×10^8	$7{\cdot}5 \times 10^{10}$	6×10^{12}
	2×10^6	1×10^{10}	6×10^{10}
	4×10^5	8×10^9	1×10^{10}
	3×10^5	7×10^9	8×10^9
	2×10^5	5×10^9	6×10^9
7	2×10^6	4×10^{10}	2×10^{11}
	2×10^5	9×10^9	2×10^{10}
	1×10^5	6×10^9	1×10^{10}
	4×10^4	5×10^9	4×10^9

Growth of bacteria from actively growing cultures

As described above, it is possible to determine the growth rate on agar of bacteria taken from fully grown cultures. However, any one determination is likely to deviate from the mean by as much as 100 % and such determinations have to be made separately for each experiment on account of uncontrollable variations in the initial lag period. Nevertheless, there was evidence that the growth of *E. coli* on nutrient agar was extremely constant, under constant conditions, and if one could only determine this rate accurately, and at the same time get rid of the initial lag period, it would no longer be necessary to make separate determinations for each experiment; all that would be required would be a knowledge of the number of bacteria plated, and the time during which growth was taking place.

Consequently some tests were made with bacteria from actively growing cultures in which the bacteria were still actively dividing. These tests showed that when bacteria were taken from a broth culture, whether aerated or not, containing more than 10^7 bacteria/ml., a short initial lag was obtained. But if the culture contained less than 10^7/ml., the growth rate was exponential from the moment of plating. Table 5 shows the results with cultures containing from 2×10^6 to 7×10^6. For each test 0·1 ml. of the culture was plated, using the undiluted culture for the 4 hr. series and a dilution of 1/60 for the 6 hr. series. The mean increase after 4 hr. growth was found to be $5{\cdot}8 \times 10^3$ times, and after 6 hr., $3{\cdot}8 \times 10^5$ times. These results indicate a generation time of 19½ min., with no initial lag period.

It is clear that the number of bacteria/plate may be estimated simply from the incubation period and the number of bacteria initially plated, and this method was adopted for the mutation rate experiments.

Table 5. *Growth of* E. coli *from actively growing cultures*

Test	Inoculum (no./ml.)	Factor of increase after 4 hr.	Factor of increase after 6 hr.
1	$2{\cdot}1 \times 10^6$	$6{\cdot}2 \times 10^3$	$4{\cdot}3 \times 10^5$
2	$2{\cdot}6 \times 10^6$	$5{\cdot}8 \times 10^3$	$3{\cdot}8 \times 10^5$
3	$2{\cdot}2 \times 10^6$	$6{\cdot}9 \times 10^3$	$3{\cdot}75 \times 10^5$
4	$3{\cdot}7 \times 10^6$	$5{\cdot}6 \times 10^3$	$4{\cdot}2 \times 10^5$
5	$2{\cdot}7 \times 10^6$	$5{\cdot}75 \times 10^3$	$2{\cdot}7 \times 10^5$
6	$6{\cdot}9 \times 10^6$	$4{\cdot}5 \times 10^3$	$3{\cdot}85 \times 10^5$
	Means	$5{\cdot}8 \times 10^3$	$3{\cdot}8 \times 10^5$

Number of phage particles required

An important condition for the success of the aerosol method of spraying phage particles on to bacterial colonies is that a sufficiently large number of phage particles must be delivered and bring about immediate infection of all or nearly all sensitive bacteria. If an appreciable number of bacteria escape immediate infection, they will be able to pass through one or more divisions before being lysed, and during this time additional mutations may occur; and, when there is an insufficient excess of phage; an unduly high mutation rate will be obtained.

Table 6. *Effect of varying amounts of phage on appearance of mutants*

No of phage particles applied	No. of bacteria/plate	Mean no. of resistant colonies/plate*
3×10^{10}	$4{\cdot}3 \times 10^8$	3·00 3·75 4·75
$2{\cdot}75 \times 10^9$	$4{\cdot}3 \times 10^8$	3·25 3·25 4·75

* Each figure is derived from observations on four plates.

An attempt was made to determine the critical excess of phage over bacteria by taking an extremely concentrated phage suspension ($1{\cdot}5 \times 10^{11}$/ml.) and spraying it in varying concentrations, each on to a series of similar bacterial cultures all containing about 4×10^8 bacteria/plate. Thus one series received about seventy times as many phage particles as bacteria and another only six times as many. From the mean numbers of resistant colonies surviving each treatment (Table 6) it will be seen that there is no difference between the two series, and consequently nothing is gained by applying to the bacteria more than a six-fold excess of phage, when the number of bacteria is about 4×10^8/plate. In other tests, a smaller number of phage particles was applied, and this sometimes resulted in a rise in the number of surviving colonies, indicating insufficient infection at the time of spraying. However, the actual point at which this phenomenon becomes important is difficult to establish, owing to uncontrolled fluctuations.

Under conditions of reasonably low humidity, it is possible to spray 0·3 ml. of a phage suspension in 2 min. on to an agar plate, without disturbing the positions of the bacterial colonies. Thus, if a phage stock has a titre of 2×10^{10}/ml. (which can be readily obtained for *T*1 in broth), the desired eight-fold excess can be obtained if the number of bacteria does not exceed 8×10^8/plate, and this latter number is just sufficient to yield a workable number of phage-resistant mutations.

RESULTS

Mutation rate after 4 and 6 hr. growth on nutrient agar at 37°

At the same time as the growth-rate determinations summarized in Table 5 were made, a similar series of nutrient agar cultures was prepared for the mutation-rate determinations. These cultures were treated in precisely the same manner as the growth-rate series, except that the process of washing and assaying was replaced by that of spraying with phage, reincubating for 48 hr. and counting the surviving colonies. Table 7 shows the results. Six actively growing cultures were prepared, on different days, and from each culture a series of platings was made, using 0·1 ml./plate at full strength for the 4 hr. series, and at a dilution of 1/60 for the 6 hr. series. This procedure leads to the development of approximately the same final number of bacteria/plate in both series. The number of bacteria plated was determined by making a careful assay of the original culture. The number of bacteria/plate at the time of the phage application was calculated by multiplying the number plated by $5·8 \times 10^3$ in the 4 hr. series, and by $3·8 \times 10^5$ in the 6 hr. series. The mutation rate is derived simply by dividing the mean number of resistant colonies by the number of bacteria/plate at time of phage application. This is legitimate because there were no mutations present on the plates at the start of the experiment, and the numbers of bacteria plated are very small in comparison with the numbers at the time of applying the phage.

The means in the two series—$0·74 \times 10^{-8}$ and $0·70 \times 10^{-8}$—agree very well, and statistical analysis by the *t* test confirms that the two means do not differ significantly. This agreement gives encouraging justification for the method adopted.

It is thus evident that no matter whether a large number of bacteria is grown for a comparatively short time (sufficient for twelve generations), or a smaller number for a longer time (sufficient for eighteen generations), the number of mutations expressed as proportion of mutants in the bacterial population is the same. One must, however, make the important reservation that this regularity can only be assured for bacteria which are growing in the exponential phase. If bacteria are taken from old fully grown cultures, or if they are allowed to grow on a plate to a density sufficient to cause a slowing down in the rate of increase, the method here described becomes unreliable.

Sub-classification of mutants

It is known that resistance to a particular phage may be produced by several more or less independent mutations (Demerec & Fano, 1945). *E. coli* has two principal mutants resistant to *T*1, namely, *B*/1, resistant to *T*1 but sensitive to *T*5, and *B*/1, 5, resistant to both *T*1 and *T*5. An attempt was made to determine the relative proportions of these and of certain other mutants in representative samples obtained by the spraying method. This part of the investigation was, however, made difficult by the property which *T*1 has of producing mutants of the type known as *T*1*h* (formerly designated 'α'), capable of lysing *B*/1 bacteria, but not *B*/1, 5 (Luria, 1945). If there were an appreciable amount of *T*1*h* in the stock of *T*1 used for spraying, most or all of the *B*/1 mutants on a plate would be eliminated. Furthermore, even if it were possible to prepare a stock of *T*1 entirely free of *T*1 *h*, mutation from *T*1 to *T*1*h* could still take place on the plates. Thus the determination of the proportion of *B*/1 mutants is always an underestimate.

Table 7. *Mutation rate after* 4 *and* 6 *hr. growth*

Culture	Age of culture (hr.)	No. of bacteria plated	No. of bacteria/plate at time of phage application	Mean no.* of resistant colonies/plate	Mutation rate/10^8 bacterial divisions
1	4	$2{\cdot}1\times10^5$	$12{\cdot}2\times10^8$	9·00	0·74
				7·75	0·64
				8·75	0·72
2	4	$2{\cdot}6\times10^5$	$15{\cdot}1\times10^8$	12·00	0·79
				16·00	1·06
3	4	$2{\cdot}2\times10^5$	$12{\cdot}8\times10^8$	7·50	0·59
				10·25	0·80
4	4	$3{\cdot}7\times10^5$	$21{\cdot}5\times10^8$	19·50	0·91
				17·00	0·79
5	4	$2{\cdot}7\times10^5$	$15{\cdot}7\times10^8$	12·00	0·76
				11·00	0·70
6	4	$3{\cdot}45\times10^5$	$20{\cdot}0\times10^8$	10·00	0·50
				11·60	0·58
1	6	$3{\cdot}5\times10^3$	$13{\cdot}3\times10^8$	11·25	0·85
				12·25	0·92
				7·50	0·56
2	6	$4{\cdot}3\times10^3$	$16{\cdot}3\times10^8$	14·75	0·90
				13·00	0·80
3	6	$3{\cdot}7\times10^3$	$14{\cdot}1\times10^8$	6·25	0·44
				9·25	0·66
4	6	$6{\cdot}2\times10^3$	$23{\cdot}6\times10^8$	13·75	0·58
				19·75	0·84
5	6	$4{\cdot}5\times10^3$	$17{\cdot}1\times10^8$	11·75	0·69
				10·00	0·58
6	6	$6{\cdot}9\times10^3$	$26{\cdot}2\times10^8$	14·00	0·53
				21·00	0·80

Mean mutation rate (4 hr.) $= (0{\cdot}74 \pm 0{\cdot}04)\times10^{-8}$.
(6 hr.) $= (0{\cdot}70 \pm 0{\cdot}04)\times10^{-8}$.
Difference between means $= 0{\cdot}04\times10^{-8}$.
Standard deviation of difference $= 0{\cdot}06\times10^{-8}$.
$t = 0{\cdot}67$, P (26 degrees of freedom) $= 0{\cdot}5$.

* Each mean is derived from observations on four plates.

Preliminary tests indicated considerable variations in the proportions of *B*/1 and *B*/1, 5 in different experiments, and in particular it was found that cultures derived from strain *B*/*r* produced a lower proportion of *B*/1 mutants than cultures derived from strain *B*. Such variations might have been due to differences in mutability of the different bacterial cultures, but equally to variations in the amount of *T*1*h* in the stocks of phage used for spraying. In order to distinguish between these alternatives, two fully grown broth cultures were set up, one of strain *B*, the other *B*/*r*, and from each of these two cultures three sub-cultures were prepared by taking a small inoculum in broth and incubating for 2½ hr. It was so arranged that each sub-culture contained actively dividing bacteria, and such a low number of phage-resistant mutants that a sample of 0·1 ml. would in practice be entirely free of mutants. Platings were made from the sub-cultures and incubated for 6 hr., when the plates were sprayed with a sample of phage containing a minimum of *T*1*h* (*c*. 30–40/ml. tested against *B*/1). After further incubation for 3 days, the mutant colonies had grown to a convenient size, and were tested for resistance to a number of phages by the streak method of Demerec & Fano (1945). From each mutant colony a broth suspension was prepared for the streak tests. Sometimes bacteria thus obtained would not grow when streaked out in concentrated suspensions, presumably due to the presence of phage mutants in the suspension. This difficulty was overcome by streaking the bacteria first in dilute suspensions and thus freeing them from the phage.

Table 8*a*. *Sub-classification of mutants derived from* E. coli *strains B and B*/*r*

	Strain *B*					Strain *B*/*r*				
	Test phage			Mutant		Test phage			Mutant	
Sub-culture	*T*3	*T*1*h*	*T*5	No.	Type	*T*3	*T*1*h*	*T*5	No.	Type
1	S	R	R	53	*B*/1, 5	S	R	R	64	*B*/1, 5
	S	S	S	6	*B*/1	S	S	S	1	*B*/1
	S	R	S	1	*B*/1*h*	R	S	S	1	*B*/1, 3
2	S	R	R	67	*B*/1, 5	S	R	R	79	*B*/1, 5
	S	S	S	19	*B*/1	S	S	S	9	*B*/1
	R	S	S	1	*B*/1, 3	R	S	S	1	*B*/1, 3
3	S	R	R	62	*B*/1, 5	S	R	R	108	*B*/1, 5
	S	S	S	31	*B*/1	S	S	S	5	*B*/1
	S	R	S	2	*B*/1*h*	R	S	S	1	*B*/1, 3
	S	S	R	1	*B*/1, 5*x*					

R = resistant, *S* = sensitive.

Each mutant was tested against the phages *T*3, *T*5 and a stock of *T*1*h* specially prepared, resistance to *T*1 being assumed. The results are shown in Tables 8*a* and *b*, and confirm that bacteria from strain *B*/*r* produce fewer *B*/1 mutants in comparison with *B*/1, 5 than do bacteria from strain *B*. There are also indications that the different sub-cultures of the same strain are heterogeneous in their capacity to mutate to *B*/1, though further data are needed to make this clear. Finally, it is seen that a number of exceptional mutants appear, which do not fit strictly in to the classes *B*/1 or *B*/1, 5. Thus there are three of the type '*B*/1 *h*', which are resistant to *T*1 *h* but sensitive to *T*5; one '*B*/1, 5*x*', which is sensitive to *T*1 *h* but resistant to *T*5, and four *B*/1, 3. The occasional occurrence of simultaneous resistance to *T*1 and *T*3 had already been recorded by Demerec & Fano (1945).

Table 8*b*. *Summary of data from Table* 8*a*

	Strain *B*	Strain *B*/*r*
Sub-culture	Ratio of *B*/1, 5 to *B*/1	Ratio of *B*/1, 5 to *B*/1
1	53 : 7	64 : 2
2	67 : 20	79 : 10
3	63 : 33	108 : 6
Total	183 : 60 (or 25 % *B*/1)	251 : 18 (or 7 % *B*/1)

It must, however, be admitted that the streak method for sub-classifying the various mutants is not very satisfactory, since there is a possibility that secondary mutations will occur during the period of growth of the mutant colonies on the plates or in the broth suspensions prepared from the mutant colonies. However, this does not alter the conclusion that resistance of *E. coli* to phage *T*1 may be brought about by any one of a number of different mutations, evidence for five of which has been obtained here, and that the relative or absolute frequency of the different mutations may vary in different bacterial cultures, and under the selective action of different phage stocks.

DISCUSSION

Luria & Delbrück (1943) were able to prove that the heritable change in a bacterium which brings about phage resistance is something in the nature of a spontaneous mutation, i.e. that it is not a change induced by the application of the phage, but occurs in the bacterium before the phage comes into contact with the bacterium. They were also able to estimate the mutation rate to phage resistance.

The technique in the experiments described here is valid only if one accepts Luria & Delbrück's hypothesis. If this is done, the estimates of mutation rate made here are more precise. The absolute value of $0{\cdot}7/10^8$ bacterial divisions is within the range of figures obtained by Luria & Delbrück. Furthermore, what is established here is the constancy in over-all mutation rate under constant conditions: i.e. by using one particular culture of bacterium and one particular culture of phage, and confining our observations to the period during which the bacteria are in an actively dividing state, we find that *mutation rate is a constant function of cell division.* This is the main conclusion to be drawn from these experiments, and gives further support to the genetic interpretation of the change from phage sensitivity to phage resistance.

The difference between the strains *B*/*r* and *B* in producing mutants *B*/1 and *B*/1, 5 may be due to some property in the *B*/*r* strain associated with radiation resistance, but it is more probably a chance effect. This is supported by the data in Table 8 *a*, which indicate that different sub-cultures from the same strain differ in the relative proportions of $B/1$ and $B/1,5$ mutants which are produced. It thus seems possible that such variations in mutability may arise spontaneously after a comparatively small number of cell generations. Whether the total mutation rate to $T1$ resistance would be materially affected by this variability in mutation rate to $B/1$ (which in these experiments amounts to a maximum of 30%) is not known; nor have we any information on the specificity of the effect, i.e. whether a high production of $B/1$ mutants is associated with a high production of some other mutants as well. Further work is required on these problems.

The author wishes to express his thanks to Dr M. Demerec for offering laboratory facilities for this investigation, to Dr Seymour Cohen for preparing the concentrated phage stock mentioned on p. 137, to Dr S. E. Luria for suggesting a number of alterations to the manuscript, and to Miss Marion Crippen for technical help.

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The Isolation and some Properties of a Virus-Inhibiting Protein from *Phytolacca esculenta*

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SUMMARY: An inhibitor of plant viruses can be isolated from the sap of *Phytolacca esculenta* by differential precipitation with ethanol followed by adsorption on celite and elution with 10% NaCl. Purified preparations contain 14–15% nitrogen and 8–12% carbohydrate and the inhibitor is probably a glycoprotein. Denaturation leads to loss of inhibiting power. The protein, unless denatured, is unaffected by pepsin and trypsin.

The glycoprotein is isoelectric at about pH 7. It can combine with tobacco mosaic virus, and when salt-free solutions of the two are mixed in certain proportions at pH values between their isoelectric points it precipitates the virus in the form of paracrystalline threads. The glycoprotein also precipitates tomato bushy stunt virus.

When added to several plant viruses, the glycoprotein causes an immediate reduction in infectivity, but has no effect on a bacteriophage. Non-infective mixtures regain infectivity when diluted. No evidence was found for a combining ratio of virus to inhibitor necessary to cause loss of infectivity. The mechanism of virus neutralization is discussed.

Substances that are known to inhibit the infectivity of tobacco mosaic virus include a polysaccharide isolated from yeast (Takahashi, 1942, 1946) and a number of proteins such as trypsin, ribonuclease, globin and clupein (Stanley, 1934; Loring, 1942; Kleczkowski, 1946). They vary in their efficiency as inhibitors and ribonuclease is the most active, but all show certain common features. Reduction of infectivity occurs immediately the virus and inhibiting substances are mixed, and infectivity can be restored by diluting the mixtures. Also all the proteins are isoelectric at pH values above 7.

Sap from a number of different plant species is also known to inhibit infectivity of tobacco mosaic virus and of some other mechanically transmissible plant viruses (Grant, 1934; Fulton, 1943; Kuntz & Walker, 1947) in much the same manner as the inhibitors named above, and of those so far studied saps from species of *Phytolacca* seem to be the strongest inhibitors. No attempts have been made to isolate the materials from plant saps responsible for inhibition, but from their studies on crude sap of spinach Kuntz & Walker (1947) conclude that there are two inhibiting principles present and that the properties of one of them suggest that it may be a protein.

This paper describes a method for concentrating and purifying the inhibitor from sap of *P. esculenta* and its preliminary identification as glycoprotein.

MATERIALS AND METHODS

The sap of different plants was obtained by passing their leaves through a domestic meat-mincer and expressing the juice through cheese-cloth. The sap was usually left for a few hours at room temperature and then clarified by centrifugation for ½ hr. at 3000 r.p.m.

Most of the inhibition tests were made with tobacco mosaic virus (T.M.V.), which was used as liquid crystalline preparations made from the sap of infected tobacco plants by the method described by Bawden & Pirie (1943). Purified preparations of tomato bushy stunt virus and potato virus *X* were also used and these were obtained from infected tomato plants by precipitation methods (Bawden, 1943). Cucumber mosaic virus and the Rothamsted culture of tobacco necrosis virus were used in the form of sap from infected tobacco plants clarified by centrifugation at 8000 r.p.m.

To assay the inhibitory effect of tested fluids, they were mixed with equal volumes of solutions of purified tobacco mosaic virus, and the infectivity of the mixtures was estimated by the local lesion method using *Nicotiana glutinosa* as a test plant. Each mixture was inoculated to at least twelve half-leaves, and the half-leaves allotted to each treatment were distributed among several plants so as to form a randomized block. Inoculations were made by rubbing the leaf surface with the forefinger wet with inoculum, and after inoculation the leaves were rinsed with water. Infection tests with tomato bushy stunt virus were also made by the local lesion method in *N. glutinosa*, with potato virus *X* in *N. tabacum* and with tobacco necrosis virus in bean (*Phaseolus vulgaris*, var. Prince). With cucumber virus tobacco was used as a test plant and inhibition shown by failure to cause systemic symptoms. Each mixture was inoculated to at least ten tobacco plants.

The inhibitory power of any one material on the same virus preparation can vary when tested on different sets of test plants, so that any critical comparison between two inhibiting materials has to be made in the same experiment.

Proteolytic and ribonuclease activity were tested by methods previously described (Kleczkowski, 1944, 1946), and phosphatase (nucleotidase) activity by a method based on estimation of inorganic phosphate released from a solution of nucleic acid previously depolymerized by incubation at pH 9. Nitrogen was determined by the micro-Kjeldahl method and carbohydrate and phosphorus colorimetrically, the carbohydrate by the orcinol-sulphuric acid method using glucose as the standard of comparison, and the phosphorus by a method based on reduction of phosphomolybdic acid by stannous chloride in the presence of sulphuric acid (Kleczkowski, 1946). When the total phosphorus content of different materials was estimated, they were first incinerated with sulphuric acid and cleared by adding a few drops of perhydrol.

Properties of the inhibitor in clarified sap

Table 1 shows the results of two experiments in which the inhibiting effect on 0·02 % tobacco mosaic virus (T.M.V.) of sap from *Phytolacca esculenta* (*P.E.*) was compared with that of several other plants. The inhibitory power of *P.E.* sap was much greater than that of sap from any of the other plants tested, and that of tobacco and tomato sap was negligible.

Sap from *P.E.* was also an effective inhibitor of tomato bushy stunt virus, potato virus *X*, tobacco necrosis virus and cucumber virus. This was also found to be true with the purified inhibitor, but no attempts were made to compare in detail the relative susceptibility of the different viruses. However, neither purified *P.E.*-inhibitor nor ribonuclease affected the activity of a bacteriophage. Tests with a bacteriophage ($S2P_{11}$) of *Rhizobium* sp. were kindly made by Dr J. Kleczkowska. Bacteriophage activity was estimated by plaque counts obtained by the poured plate method (Kleczkowska, 1945). A crude bacteriophage culture was diluted 10^{-5} in the sterile liquid medium containing the isolated *P.E.*-inhibitor or ribonuclease at various concentrations between 0·05 and 0·001 %. The fluid was then mixed with 24 hr. liquid cultures of the host bacteria, and the mixture was added to melted agar, cooled to 42°, and plated. Equal numbers of plaques (about 400 per plate) were produced on the control plates and on those containing *P.E.*-inhibitor or crystallized ribonuclease.

Table 1. *The effect of sap from different plants on the infectivity of tobacco mosaic virus*

Sap from	Dilution of sap	Average no. of lesions/leaf
	Experiment 1	
Phytolacca esculenta	1/10	0
	1/100	5
	1/1,000	33
	1/10,000	87
Datura stramonium	1/1	2
	1/50	58
Tobacco	1/1	29
	1/50	92
Tomato	1/1	24
	1/50	84
Water control		89
	Experiment 2	
Phytolacca esculenta	1/50	0
	1/500	4
Spinach	1/1	0
	1/50	10
Sugar beet	1/1	1
	1/50	10
Water control		70

Table 2. *The recovery of infectivity by diluting a mixture of tobacco mosaic virus and sap of* Phytolacca esculenta

	Average no. of lesions/leaf obtained with mixtures diluted in water to			
Mixture	1/1	1/10	1/100	1/1000
1 vol. 0·005 % T.M.V. + 1 vol. sap. dil. 1/10	0	3	6	4
1 vol. 0·005 % T.M.V. + 1 vol. H_2O	110	64	20	4

The inhibition of plant viruses occurred immediately the sap of *P.E.* and virus solutions were mixed and there was no further fall in infectivity when the mixtures were incubated at room temperature or at 37°. Diluting non-infective mixtures in water restored infectivity of the virus (Table 2).

The sap not only inhibited infectivity when it was mixed with a virus solution and the mixture was inoculated to test plants, but also when it was first rubbed over the leaves, which were then rinsed with water, dried with blotting paper and the virus solution inoculated immediately after. The reduction in infectivity produced by the two methods with a purified preparation of the inhibitor is shown in Table 3. At first sight the results suggest that inoculation with the inhibitor first is as effective as inoculation of a mixture of the virus and inhibitor. However, it will be seen that a previous rubbing of the leaves with water alone considerably reduces the number of lesions produced, and this needs to be taken into consideration when comparing the results of tests in which leaves were rubbed once or twice.

Table 3. *The effect of inoculating the inhibitor and tobacco mosaic virus separately and together*

First inoculation	Second inoculation	Average no. of lesions/leaf
1 vol. 0·0002 % inhibitor +1 vol. 0·015 % T.M.V.	None	4
0·0001 % inhibitor	0·0075 % T.M.V.	3
0·0075 % T.M.V.	0·0001 % inhibitor	10
Water	0·0075 % T.M.V.	17
0·0075 % T.M.V.	Water	34
0·0075 % T.M.V.	None	47

The inhibitory power of the sap was unaffected by heating for 10 min. at 70°; but was diminished by 10 min. at 80° and completely destroyed by 10 min. at 100°. Drying the sap in a desiccator at 37° and suspending the dry residue in the original volume of water did not affect the inhibitor; nor did freezing and thawing, changes of pH between 2·5 and 8·0, or dialysing the sap in a cellophan bag against distilled water, or centrifugation for 1 hr. at 40,000 r.p.m. Filtration through a porcelain filter L_1 did not affect its inhibiting power, but subsequent filtration through an L_3 or L_5 filter considerably diminished it.

Sap expressed from minced leaves of *P.E.* is viscous and rather frothy. After keeping for 24 hr. at room temperature and centrifuging for ½ hr. at 3000 r.p.m., the clarified sap contains about 7 % of dry matter. Much inactive material can be coagulated and removed by heating the sap to 60–70°, freezing and thawing, or precipitation with half a volume of 96 % ethanol.

The inhibitor is precipitated quantitatively by adding two volumes of ethanol to one volume of the sap and can be redissolved completely in a 2 % NaCl solution. The precipitate produced by ethanol, however, must be taken up in 2 % NaCl within 1 hr., for if the time is prolonged the inhibiting power of the resulting solution is progressively diminished. If water is used instead of 2 % NaCl, only part of the inhibitor is recovered.

The inhibitor begins to be precipitable by ammonium sulphate when the concentration reaches half-saturation, and progressively more is precipitated as the concentration of the salt increases. Precipitation is almost complete when one volume of the sap is mixed with twenty volumes of saturated ammonium sulphate solution. The precipitated inhibitor dissolves readily in water.

Isolation of the inhibitor based on fractional precipitation with ethanol and ammonium sulphate, combined with dialysis against distilled water and adjusting the pH to various values, gave concentrated preparations, but usually a number of fractions, all with some inhibitory power, were obtained. The fractions differed from each other in various respects and were not homogeneous, as they could usually be fractionated still further. They contained 20–60 % of carbohydrate and had no detectable proteolytic or ribonuclease activity, but they were invariably rich in a phosphatase (nucleotidase) having a maximal activity between pH 5 and 6.

In preparations concentrated by precipitation, phosphatase and inhibitor were so closely associated that it seemed probable that the enzyme was the inhibitor. Eventually, however, it was found that the inhibitor but not the phosphatase was readily adsorbed from salt-free solutions by a number of adsorbents. Of the various adsorbents tested charcoal and celite (diatomaceous silica Filter-Aid, Johns-Manville) were found to be the most effective. It was not possible to elute the inhibitor from the two adsorbents with distilled water, but from celite it could be eluted with 10 % NaCl solution, though not from charcoal.

The method of isolation of the inhibitor

Of many methods of isolation tested the following was finally adopted as the most reliable in producing apparently homogeneous preparations. Five hundred ml. of 96 % ethanol are added to 1 l. of sap, and the resulting bulky precipitate is removed by centrifugation. To the clear, brown supernatant liquid 1500 ml. of ethanol are added, and the precipitate, which contains the inhibitor, is centrifuged down. The precipitate is suspended in 250 ml. of 2 % NaCl, and the undissolved material is removed by centrifugation. The clear greyish supernatant liquid is dialysed in a cellophan bag for 2 days against distilled water. The liquid, which is usually slightly acid, is then neutralized, and some insoluble material produced during dialysis is removed by centrifugation. This involves some loss of the inhibitor, usually less than 25 %, probably because it is adsorbed on the insoluble material. Adjusting the pH to 7 decreases the amount lost. Separation of insoluble material, and consequently loss of inhibitor, reaches its maximum when the pH is around 4·5.

At this stage the liquid contains about 0·5 % of dry matter, of which 50 % is carbohydrate and 8 % nitrogen. The liquid gives a strongly positive biuret test and has strong phosphatase activity. Celite is next added to the liquid, 2 g. to each 100 ml. The mixture is kept for 1 hr. at room temperature with occasional shaking, and the celite removed by filtration through a filter-paper on a Buchner funnel. In this way almost all the inhibitor is removed from the liquid, although the solid content of the liquid is reduced by less than 10 % and its phosphatase activity remains almost unchanged. The celite is washed in the funnel with 200 ml. of distilled water and suspended in 30 ml. of 10 % NaCl solution. The suspension is kept for 4 hr. at room temperature with occasional stirring, and the celite is again filtered off. The water-clear filtrate is dialysed in a cellophan bag for 24 hr. against frequently changed distilled water. A slight turbidity sometimes appears during dialysis, and is removed by filtration. About 20 % more inhibitor can be obtained from the celite by repeating the elution.

The yield of purified material from 1 l. of sap varied from 20 to 60 mg. Inhibition by a 0·0002 % solution of the purified preparation was approximately the same as that of sap diluted 1/100. Thus, assuming that the inhibitory power of the material is unaffected during purification, its initial content in crude sap is about 200 mg./l. and 60–90 % is lost during purification. However, as exposure to ethanol has a deleterious effect on the inhibitor, it may be that the recovery is greater than this but that the purified product is, weight for weight, less active than the inhibitor in crude sap.

When the purification procedure was carried out on tobacco sap, which does not inhibit infectivity of T.M.V. to any appreciable extent (see Table 1), nothing was recovered in the elute from the celite, showing that the method is selective.

Properties of the purified inhibitor

The nitrogen content of purified products varied from 14 to 15 % and the carbohydrate content from 8 to 12 %. No phosphorus was detected in any preparation. The material gives a positive biuret test, is precipitated by 95 % saturated ammonium sulphate solution and by 5 % trichloroacetic acid. When salt-free solutions are boiled, they become slightly opalescent, while in the presence of 1 % NaCl a coagulum separates. These properties all indicate that the material is a protein. Since most of the carbohydrate is not separated from the protein by precipitation with ammonium sulphate or trichloroacetic acid, it appears that the two are combined as a glycoprotein, and the inhibitor will be referred to as such.

Table 4. *Relative inhibitory powers of ribonuclease and the glycoprotein*

	Concentration (%)	Average no. of lesions/leaf
Ribonuclease	0·05	1
	0·001	4
	0·0002	15
Glycoprotein	0·0002	16
Water control		90

The fluids were mixed with equal volumes of 0·015 % T.M.V.

No critical tests for homogeneity were made, and it may be that the purified preparation contains more than one protein. However, there is a good deal of evidence connecting the inhibitor with the glycoprotein that forms at any rate the major constituent of the preparation. Of the various inhibiting substances previously tested, ribonuclease is by far the most efficient (Loring, 1942). Table 4 shows the results of an experiment comparing the inhibitory power of the purified glycoprotein from *P.E.* and that of crystallized ribonuclease prepared by Kunitz's (1940) method. It will be seen that, weight for weight, the two have similar inhibitory powers, so that if the inhibition produced by the preparation from *P.E.* is brought about by a minor component, then this must possess a much higher order of activity than ribonuclease. Again, processes that denature the glycoprotein—heating, trichloroacetic acid, or exposure to ethanol—also cause a proportional loss of inhibitory power. The glycoprotein is not hydrolysed by pepsin or trypsin, unless it has been previously denatured, and the inhibiting power similarly is not diminished by these enzymes. Drying neither denatures the glycoprotein nor affects its inhibiting power.

Denaturation of the glycoprotein by ethanol is slow, and may be demonstrated by the loss of solubility in water. The addition of two volumes of 96 % ethanol to one volume of salt-free solutions of the glycoprotein, produces an opalescence, but flocculation occurs only in the presence of salt. After precipitation with ethanol a proportion of the glycoprotein usually becomes insoluble in water, the proportion increasing with increasing time of contact with ethanol, so that after a few days all the glycoprotein becomes insoluble. The stability of suspensions of ethanol-denatured glycoprotein depends on the pH and this property was used to find its isoelectric point.

When suspensions containing 0·05 % of the glycoprotein were adjusted to different pH values (measured with a glass electrode) with HCl or NaOH, it was found that they flocculated in a few minutes at pH values between 6·7 and 7·3, but they remained stable for several hours at pH values outside this range. From this it was concluded that the isoelectric point of denatured glycoprotein is near pH 7. It is known that denaturation can alter isoelectric points of some proteins, but the alteration is usually slight and does not exceed 0·5 of the pH unit (Neurath, Greenstein, Putnam & Erickson, 1944). Thus the isoelectric point of the undenatured glycoprotein, which is soluble over a wide pH range, is probably also about pH 7, a feature which it shares with other proteins, such as ribonuclease and trypsin, which inhibit infectivity of T.M.V.

The isoelectric point of salt-free solutions of T.M.V. is at pH 4·2 (Bawden & Pirie, 1937), so that between pH 4·2 and pH 7 the glycoprotein and the virus are oppositely charged. Some other pairs of proteins that are oppositely charged precipitate one another when mixed (Kleczkowski, 1946) and this also occurs with the glycoprotein and T.M.V. When the two are mixed in salt-free solutions at pH values between their isoelectric points, they combine, mutually discharge one another and produce a visible precipitate.

No attempts have been made to determine the minimum concentrations at which T.M.V. and the glycoprotein must be mixed to give a visible precipitate, but precipitation is pronounced with 0·01 % solutions of the two. Precipitation only occurs when the ratio of the weight of virus to that of glycoprotein is between 1 : 1 and 1 : 2. Outside this range, the fluids merely become opalescent and develop a satin-like sheen, the intensity of which decreases the further the ratio of the two components departs from that causing precipitation. The addition of NaCl up to 1 %, or changing the pH to below 3 or above 7, causes immediate solution of precipitate or disappearance of opalescence. Under the microscope the precipitates are seen to consist of long, curvilinear, birefringent threads, similar to those formed in mixtures of T.M.V. with ribonuclease, clupein or serum globulin (Bawden & Pirie, 1937; Loring, 1942; Kleczkowski, 1946). Precipitation also occurs in mixtures of salt-free solutions of glycoprotein and tomato bushy stunt virus at pH values between 5 and 6·5. Using the glycoprotein at 0·005 %, precipitation was optimal when the concentration of tomato bushy stunt virus was 0·01 %. Microscopically the precipitate appeared to be granular, but no definite crystals could be identified.

Mixtures of equal volumes of 0·05 % glycoprotein and 0·05 % clupein sulphate became slightly opalescent at pH 9 but not at pH 6. The isoelectric point of clupein is at pH 12 (Miyake, 1927), so that pH 9 is in the inter-isoelectric zone, whereas pH 6 is not.

No opalescence or flocculation was observed when sap from ***P.E.***, clarified by heating at 70° and centrifugation, was mixed with equal volumes of solutions of T.M.V. at different concentrations. Tests were also made using dialysed sap to avoid the possibility that precipitation was being prevented by inorganic salts, but again there was no precipitation. The negative result cannot be explained by assuming that the concentration of the glycoprotein in the sap is too low to cause precipitation, as it is higher than 0·005 %, at which the isolated glycoprotein precipitates the virus. Dialysed sap contains 5–7 % of dry matter and only 0·01–0·02 % of the glycoprotein, so that some of the non-dialysable constituents of sap seem to act as stabilizers and prevent the virus from being precipitated.

By means of ultracentrifugation it was possible to show that T.M.V. and the glycoprotein combine when they are mixed at ratios which cause no opalescence or precipitate. These tests also produced further evidence identifying the glycoprotein with the inhibiting entity. When 0·1 % solutions of the glycoprotein were centrifuged for 1 hr. at 40,000 r.p.m., the glycoprotein was not sedimented nor was the inhibiting power of the fluid reduced. When similar solutions containing 3 mg./ml. of T.M.V. were centrifuged, however, almost all the glycoprotein and the inhibitor were removed from the supernatant fluid. Some of the glycoprotein with its inhibiting power could be recovered from the virus by dissolving the pellet in water, and precipitating the virus with one-third saturated ammonium sulphate solution. After removing the virus by centrifugation at low speed, the glycoprotein was present in the supernatant fluid with its original inhibiting power.

By analogy with toxin-antitoxin reactions, it seemed possible that inactivation of the virus by the glycoprotein might be based on combination between equivalent quantities of the two. To test this possibility, attempts were made to find the highest concentrations of T.M.V. solutions whose infectivity could be completely inhibited by solutions of the glycoprotein at different concentrations. Table 5 gives the results of an experiment in which the neutralizing power of four different concentrations of glycoprotein was tested against different concentrations of the virus. Inactivation was considered as complete in mixtures that produced an average of less than one lesion per inoculated half-leaf, and the results are given as the highest concentrations of the virus inhibited to this degree by the glycoprotein at four different concentrations.

It can be seen that as the concentrations of the virus increased, the ratio of glycoprotein to virus needed to give complete inactivation decreased. Within the range tested, the virus concentration was proportional to the third power of that of the glycoprotein. This result differs strikingly from that obtained with toxins and antitoxins, and shows that the higher the virus concentration, the smaller was the amount of the glycoprotein that was needed to neutralize each unit weight of the virus. There are clearly no fixed equivalent quantities of the virus and glycoprotein which combine before the virus is rendered non-infective.

Table 5. *The ratio of inhibitor to virus required to give complete inhibition at different virus concentrations*

Concentration of the inhibitor (mg./ml.) x	Concentration of T.M.V. (mg./ml.) y	x/y
0·024	0·72	0·03
0·012	0·09	0·13
0·006	0·01	0·60
0·003	0·0015	2·00

The relationship between y and x can be expressed by the equation $y = 51290x^3$.

DISCUSSION

From our results it is clear that sap from *Phytolacca esculenta* contains a protein that is not present in the sap from tobacco plants. The evidence suggests that it is a glycoprotein and that it is the substance in sap of *P. esculenta* responsible for the inhibition of infectivity of plant viruses. Weight for weight it is as active as an inhibitor as ribonuclease, and its identification adds a new type of substance to those known to inhibit plant viruses. How these varied substances produce their effect is uncertain, but superficially at least they appear to act similarly.

Since the inhibitory effect of trypsin was discovered (Caldwell, 1933), there has been much speculation on the mechanism of its action, some workers maintaining that it acts on the virus and others that it acts on the host plant. The fact that an inhibitory material used at constant concentration caused the same relative reduction of infectivity irrespective of virus concentration, was often considered as evidence that the inhibiting material affected susceptibility of the host plant (Chester, 1934; Stanley, 1934; Caldwell, 1938).

The results with the glycoprotein, in addition to those with some other of the inhibiting substances (Kleczkowski, 1944, 1946), show that the inhibitors do combine with tobacco mosaic virus, so that the simplest explanation of the phenomenon would be that in combining with the virus the inhibitors block some groups on it that are essential for the establishment of infection. However, there are various other facts that do not fit in with this view of a simple quantitative neutralization of the virus. First, of the substances that can combine with and precipitate T.M.V., not all are equally active as inhibitors, and some, such as clupein and globin, have only little neutralizing power.

Hence mere combination of a protein with the virus is not enough to explain inhibition, unless different substances combine with and block different groups. Secondly, if inhibition were dependent on, and a consequence of, combination with the virus, it is to be expected that for complete neutralization of infectivity a given weight of virus would need to combine with a certain minimum quantity of inhibitor, but there is no evidence for such a neutralizing ratio. On the contrary, there is good evidence that no such ratio exists. This is partly implied by the development of infectivity when non-infective mixtures of virus and inhibitor are greatly diluted, for dilution leaves the ratio of the two unchanged. However, this is not conclusive, for if the combination is reversible, dilution could disrupt the combination and leave free virus particles. More positive evidence for the lack of a neutralizing ratio is supplied by the fact that the higher the virus concentration, the smaller is the ratio of combined inhibitor to virus that coincides with complete neutralization. Hence, there is no reason to assume that the combination between the two is at all essential for inhibition to occur. This suggests an effect on the host rather than on the virus, a suggestion supported by the fact that the number of lesions produced is reduced if the leaves are inoculated first with the inhibitor alone. In our present state of knowledge of the processes involved in the establishing of infection, any attempt to distinguish between effects on the virus and on the host is probably premature. All that can be said with certainty is that the virus and inhibitors can combine, and that the presence of the inhibitors prevents infection. It is also possible that the inhibitors can combine with some constituents of the host cells, but this has not been demonstrated.

Many examples are known of substances that are structurally similar being biologically antagonistic. The inhibitors could be further examples of such biological antagonism, acting either because they resemble some cell constituents with which the viruses must combine to establish infection or because they themselves combine with these constituents and so prevent the virus from doing so. Although this is a possibility that needs further study, the wide range of substances that can act as inhibitors ranging from carbohydrates, through various enzymes, to the glycoproteins, does not suggest that any great structural specificity is involved. A lack of specificity is also apparent in that substances such as the glycoprotein from *P. esculenta* and ribonuclease are effective inhibitors of all the plant viruses against which they have been tested. However, neither substance affected the activity of a bacteriophage, suggesting that the inhibitors may act specifically against some phase of the infection process essential for the establishment of mechanically transmitted plant viruses but absent from the process of infection of bacteria by bacteriophage.

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The Bacteriophages of *Pseudomonas pyocyanea*

1. The Effect of Various Substances upon their Development

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SUMMARY: Bacteriophages were isolated from several strains of *Pseudomonas pyocyanea* and were assayed against an indicator strain. Counts of up to 10^{10}/ml. were obtained in both broth and a defined medium. The phages are stable at 60° but rapidly inactivated at 65°. They are unaffected by 50% glycerol and are stable at pH 6·5–10·0 but not below 6·5.

About 500 compounds have been tested for their inhibitory action on the development of these bacteriophages. All the sulphonamides, amidines, pyrimidines, organometallic compounds, plant extracts, mould cultures and antibiotics tested were inactive at concentrations permitting the growth of the host. Proflavine exerted a viristatic effect while notatin and hydrogen peroxide were lethal in a defined medium but not in broth.

As part of an investigation into the chemotherapy of virus diseases, the bacteriophages of *Ps. pyocyanea* and, in particular, the effects of various substances on the bacteriolytic action of these phages have been studied. Whilst it is recognized that information obtained in such work may not be applicable to virus infections of animals, the direct investigation of the chemotherapy of these infections on a large scale presents such difficulties that preliminary investigations were made with bacteriophage as a test virus. Bacteriophages have also been used in this manner by Jones (1945), Klein, Kalter & Mudd (1945), Fitzgerald & Babbit (1946) and Fitzgerald & Lee (1946). It was felt that the use of a relatively resistant type of host, not nutritionally exacting, such as *Ps. pyocyanea* would offer decided advantages. This organism grows rapidly in synthetic media and active phage preparations can be obtained from such cultures. It is, therefore, a convenient host to use for more fundamental work on bacteriophage metabolism.

The lytic phenomena associated with *Ps. pyocyanea* were described by Hadley (1924). Fastier (1945) isolated one specific bacteriophage, and Bordet & Bordet (1946) studied the close relationship of the lytic agent to bacterial variation.

EXPERIMENTAL

Strains. Ten strains of *Ps. pyocyanea* were investigated. Six (nos. C1–C6) were originally isolated by Dr F. R. Selbie of the Bland Sutton Institute of Pathology from war wounds, and four (nos. C7–C10) were isolated in our own laboratory. Strains C1, C2, C3, C5 and C8 were characterized initially by marked pyocyanin production and a silvery sheen on the surface of confluent growth, typical of phage activity on this organism. These strains are referred to below as 'lysogenic', implying only that they can be subcultured without loss of visible phage activity; presumably sensitive cells serving to maintain the phage are present. True lysogenesis, as defined by Delbrück (1946), does not entail lysis of the host. By rapidly repeated subculture in broth, however, phage-resistant strains were obtained. The lysogenic strains gradually lost their capacity to produce pyocyanin, unless it was maintained by passage through mice. At no time in two years' study did strains C4, C6 and C10 produce pyocyanin or show any evidence of phage action. This does not necessarily indicate that phage was not carried by these strains but rather that we found no bacteria susceptible to them. All ten strains produced fluorescin and retained this capacity throughout the investigation. Culture C9 was not studied in detail; its phage appeared to develop only after storage and even then its appearance was erratic.

Maintenance of strains. Cultures were grown at 37° on nutrient agar, in nutrient broth, or in a defined medium composed of lactic acid 2·4 g., NaCl 5·0 g., $(NH_4)_2HPO_4$ 1 g., KH_2PO_4 1 g., $MgSO_4.7H_2O$ 0·2 g., and distilled water to 1 l., the pH being adjusted to 7·0 with NaOH and the medium sterilized for 15 min. in the autoclave at 15 lb. steam pressure. Stock cultures were subcultured at monthly intervals on agar and some were stored in the freeze-dried state. It was found advantageous to pass the lysogenic strains through mice occasionally, but passage through the yolk sac of 9-day chick embryos was a more satisfactory means of maintaining these strains and their associated phages, without the risk of contamination.

Cross-reactions of culture filtrates. In order to test the possibilities of cross infection by the phages of different strains, bacteria-free filtrates from 24 hr. broth cultures of each strain were 'spotted' on agar-plate cultures of all other strains not showing visible evidence of phage action (C4, C6, C10 and resistant variants of C1, C3, C5 and C8). The filtrates were designated by the number of the host preceded by the letter F, and individual phages isolated from the filtrate, by Pa, Pb. F1, the filtrate from C1, showed pronounced activity against C10 and C7, whilst F3, from C3, was moderately active against C10. No other cross reactions were observed, so that quantitative work was limited to the action of these filtrates on C10.

When F1 was grown on C10, a high titre filtrate was obtained; this gave rise to two distinct types of plaque (Pl. 1), a large one with a halo (Pa-type), and a smaller clear one (Pb-type). Four consecutive single plaque isolations were made from each of these two types. When grown on C10 in broth, Pa and Pb filtrates gave plaque counts up to 10^{10}/ml., but complete lysis of C10 never occurred. Pb filtrates gave only Pb-type plaques but Pa filtrates did not invariably give plaques of one type, although the Pa-type always greatly predominated. These phages were maintained as Seitz filtrates. It was not possible to produce a lysogenic strain of C10 by implanting F1 in it either *in vitro* or *in vivo* (mouse passage). This was however accomplished with F1 on C7, yielding the lysogenic strain C7X.

Titration of phages Pa and Pb against strain C10. The method of titration finally adopted was to mix 1 ml. of a series of a hundred-fold dilutions of the phage filtrate, or lysed culture, in Ringer's solution with 0·5 ml. 24 hr. broth culture of C10, and add 2·0 ml. warm nutrient agar. After shaking, the tube was 'sloped' and examined after 18–24 hr. incubation at 37°. This method was much more rapid and convenient than the plate assay used by many workers. In titrations of F1 grown on C10, small numbers of C10 in the material did not matter, since excess of C10 was added in the course of the assay. Of the lysogenic strains, only C1, C3 and C7X possessed phages that could be titrated against C10.

Preparation of filtrates. Bacteria-free Seitz filtrates of 24–72 hr. broth cultures gave counts of up to 10^{10}/ml. for Pa and Pb phages. Seitz filtrates of cultures in defined media were, however, inactive. When Gradacol membranes of 500 mμ porosity were used, the loss was still considerable, but counts of 10^6/ml. were obtained. In the case of Pa, membranes of pore size 100 mμ. allowed some phage to pass, but counts were diminished from 10^6/ml. to less than 100/ml.

Properties of Pa bacteriophage

Heat stability. Type Pa filtrate (6×10^9/ml.) in nutrient broth, in Ringer's solution and in lactate medium was stable between 50 and 60°, counts being only slightly diminished after 30 min. It was, however, inactivated within 10 min. at 65°. This heat stability was characteristic of all strains which could be assayed and was the basis of a convenient method of obtaining active phage from lysogenic strains, since the host cells were much less resistant; temperatures of 60° for 30 min. usually killed the host, provided the cell population was not very large.

Effect of glycerol. Glycerol was useful as a means of obtaining phage preparations from lysogenic strains, especially in lactate medium, because in a concentration of 50% it killed the host cells of 24 hr. cultures of C7X and C1 within 7–10 days at 37° leaving the phage unaffected. At 0° the host cells were not killed within a month.

Effect of pH. Type Pa filtrate was kept for 24 hr. at 37° in phosphate and citric acid buffers (pH 2·2–8·0), and in boric acid KCl-NaOH buffers (pH 8·0–10·0). At pH 2·2–4·0 there was complete inactivation within 3 hr. and the count was very much diminished at pH 5·0 and 6·0. At pH 7·0–10·0 (in steps of 0·5 pH unit) there was a slight initial fall in every case, followed by a remarkable stability for at least 24 hr. The initial fall was quite common and occurred in all media, but after this the preparations could be kept for weeks at room temperature without any significant loss of activity, provided the pH was not below 6·5. Preparations of Pa phage in broth, lactate medium, and Ringer's solution at pH values about 7·0–7·5 were kept for several months at room temperature and maintained their titres.

Testing of drugs

In a preliminary test, the action of the drug was studied directly on agar plates and any substances found promising were tested by two quantitative methods.

Agar-plate test, carried out in the presence of multiplying host cells. 1 ml. amounts of a ten-fold series of drug dilutions were mixed with 9 ml. nutrient agar and plates poured. (On 'synthetic' lactic agar the phage action was very difficult to assess.) The plate was marked into four divisions. Three different lysogenic strains showing pronounced and visible phage activity were sown each on one-quarter of the plate. The fourth quarter of the plate was sown with C10 and a loopful of two active filtrates Pa and Pb were 'spotted' on it. After 24 hr. at 37° the lysogenic cultures were examined for silvery sheen and the C10 for lysis. The results were classified as follows.

Class A. Active: phage inhibited by less than 1/100, the drug concentration required to inactivate the host organism.

Class B. Slightly active: phage inhibited by 1/10–1/100, the drug concentration required to inactivate the host organism.

Class C. Inactive: no inhibition of phage at the highest concentration of the drug permitting growth of the host.

*Titration of phage inhibitors in actively growing cultures. Filtrate F*1 *on Culture* 10. A 24 hr. culture of C10 was diluted 1/10 in broth or lactate and 1 ml. F1 filtrate, containing 1000–10,000 phage particles/ml., was added to 9 ml. culture. After standing for 10 min. at room temperature a suitable series of concentrations of the test substance in 100 ml. bottles of the test medium were each sown with 1 ml. of the phage-host suspension, the maximum concentration permitting good growth of the host being determined beforehand. The test mixture was incubated at 37°. The phage content was determined at intervals up to 24 hr. by removing 1 ml. samples which were assayed unfiltered. This gave a figure for free phage plus phage in or on living host cells in the test bottles. Bacterial counts were made at the same time, and variation in phage counts were considered significant only if the host count were not itself influenced by the drug.

Lysogenic strains. 1 ml. of a 24 hr. culture of C1 or C7X was added to 100 ml. test medium and plaque counts of the filtered material assayed at intervals against C10. This count, therefore, determined only *free* phage passing the filter. Defined lactate media were filtered through Gradacol membranes of 500 mμ. porosity.

All tests were duplicated at different times and controls without drug were included in every experiment. After 24 hr. counts on the unfiltered material for both media were always between 10^7 and 10^{10}/ml., but values for the lysogenic culture controls were of the order of 10^6/ml. for filtered material. In the broth titration the substances tested must affect the multiplying phage in the presence of its living host, but the actual assay was carried out at dilutions at which the drug could have no effect on the phage.

Titration of phage inhibitors. Contact test carried out in the absence of host cells. Compounds found to inhibit phage in growing cultures were tested by adding a series of dilutions of the drug to phage filtrates at 37°, in different media, and for varying periods. Subsequent counts indicated whether the drug were lethal.

RESULTS

Inhibition of phage multiplication

Agar plate test. About 500 substances were tested on agar plates against lysogenic strains; the great majority were inactive in the dilutions used (Table 1). The substances tested can be roughly classified as follows: sulphonamides and related compounds, amidines, pyrimidines and other heterocyclic compounds, organo-metallic compounds, antibiotics, plant extracts, mould cultures, and a miscellaneous group of substances thought for one reason or another to be worth while investigating.

Table 1. *Action of various substances against bacteriophages of lysogenic strains of* Ps. pyocyanea *in agar plate test*

Active	Inactive
Phenol	Fifty plant extracts
Agaric acid	Fifty mould cultures
Hexylresorcinol	Penicillin (100 u./ml.)
5-aminoacridine	Notatin*
2:7-diaminoacridine	Gramicidin
2-chloro-5-amino-7-methoxyacridine	Streptothricin
Proflavine	Streptomycin
	Patulin
	Organo-metallic compounds
	Phenanthridines
	Sulphonamides
	'Homosulphonamides'
	Formaldehyde

* In presence of 0·5% glucose.

Several dyes, such as eosin and brilliant green, showed class A activity against lysogenic strains but results were difficult to read, and the dyes were not investigated further in view of other work of greater promise. One mould appeared very active when the living mould was grown on the plate, but a culture filtrate was entirely inactive. This phenomenon has been noted by Jones & Schatz (1946) in the course of similar studies on staphylococcus and *Bacterium coli* phages. The acridines were the most active class of compound; 5-amino, 2:7-diamino, 2-chloro-5-amino-7-methoxyacridines and proflavine were all active both against lysogenic strains and in the spot tests. Since very active Pa and Pb filtrates were used, small degrees of inhibition were not detectable. Thus, phenol and all the other compounds showing class A activity were inactive by the spot test, but when Pa filtrate was tested in the presence in the assay plate of, for example, 5-amino-acridine, it was obvious that concentrations of 10^{-4} and 10^{-5} of the drug not only diminished the phage count, as compared with the control assay, but rendered the plaques very hazy and very difficult to see. It was partly because of this difficulty in assessing results by the agar plate test that the quantitative tests were developed to show whether compounds were inhibiting phage reproduction. The great majority of compounds could obviously be ruled out at once as inactive by the agar plate test.

The titration of drugs (Table 2) include some of the compounds classed as active in the agar plate test and of these, only proflavine was active. It is interesting to note that several acridines were found active against *Bact. coli*

Table 2. *Action of various substances on bacteriophage in growing cultures*

Substance	Medium	Strain	Approximate phage titre/ml.		
			At 0 hr.* (test and control)	At 24 hr.	
				Test	Control
Phenol					
10^{-4}	Broth	F1 on C10	10^{0} +	10^{-10} +	10^{-10} +
10^{-4}	Lactate	C7X (lysogenic)	10^{-4} +	10^{-6} +	10^{-6} +
10^{-4}	Broth	C1X (lysogenic)	10^{-3} +	10^{-6} +	10^{-4} ++
5-aminoacridine					
10^{-4}	Broth	F1 on C10	10^{0} +	10^{-7} ++	10^{-7} ++
10^{-4}	Lactate	C7X (lysogenic)	10^{-4} +	10^{-6} +	10^{-6} +
10^{-4}	Broth	C1X (lysogenic)	10^{-3} +	10^{-6} +	10^{-4} ++
Agaric acid					
10^{-4}	Broth	F1 on C10	10^{0} +	10^{-10} +	10^{-9} +
Hexylresorcinol					
10^{-4}	Broth	F1 on C10	10^{0} +	10^{-9} +	10^{-9} +
Proflavine					
10^{-4}	Broth	F1 on C10	10^{-2} +	10^{-2} +	10^{-8} +
10^{-4}	Lactate	F1 on C10	10^{-4} +	10^{-2} +	10^{-8} +
5×10^{-4}	Broth	C7X (lysogenic)	10^{-2} +	10^{-6} ++	10^{-8} +
5×10^{-4}	Broth	C1X (lysogenic)	10^{-2} +	10^{-8} +	10^{-10} +
Patulin					
10^{-5}	Lactate	F1 on C10	10^{-2} ++	10^{-8} ++	10^{-8} ++
Notatin					
10^{-7}	Lactate + 0·5% glucose	F1 on C10	No growth of host		
10^{-8}	Lactate + 0·5% glucose	F1 on C10	10^{-2} ++	10^{0} Nil	10^{-8} ++
10^{-8}	Lactate	F1 on C10	10^{-2} ++	10^{-8} ++	10^{-8} ++
Streptomycin					
1 u./ml.	Lactate	F1 on C10	10^{0} ∞	10^{-6} ∞	10^{-6} ∞
Hydrogen peroxide					
0·0002% v/v	Lactate	F1 on C10	10^{0} +	10^{0} Nil	10^{-4} +
0·002% v/v	Lactate	C1 (lysogenic)	10^{0} ++	10^{-6} +	10^{-6} +
0·02% v/v	Broth	F1 on C10	10^{0} +	10^{-6} +	10^{-6} +
0·02% v/v	Broth	C1 (lysogenic)	10^{0} ++	10^{-6} +	10^{-6} +

Key to plaque counts: ∞ = more than 500; ++ = 100–500; + = less than 100.
* Calculated from amount of phage added.

phage by Fitzgerald & Babbit (1946) and Fitzgerald & Lee (1946). Although proflavine prevented the multiplication of F1 on culture C10, it had little, if any, effect, as judged by 24 hr. phage counts, on the lysogenic cultures C7X and C1. Notatin, although inactive in the agar plate test (see below) was markedly active against F1 in 0·5% glucose-lactate medium. It is known to be a glucose aerodehydrogenase, exerting its high *in vitro* antibacterial action by producing hydrogen peroxide in the presence of glucose and oxygen (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1945). Its inactivity in lactate medium without glucose suggested a similar mode of action against bacteriophage and results with hydrogen peroxide are in accord with this viewpoint.

Contact test. Contact tests were carried out with notatin and hydrogen peroxide in various media. A 10^{-6} concentration of a dried notatin preparation in lactate medium containing 0·5% glucose, completely inactivated a Pa preparation with an initial content of 10^8/ml., in 3 hr. at 37° and a 10^{-9} concentration inactivated it in 24 hr. Phage counts did not fall in lactate medium plus notatin, lactate medium plus 0·5% glucose, broth plus notatin and glucose broth plus notatin. Similarly, hydrogen peroxide in a concentration of 0·02% (v/v) inactivated Pa in lactate medium within 3 hr., but even 2% (v/v) had no effect in broth. There is no explanation, at present, for this lack of action in broth.

As with proflavine, neither notatin nor hydrogen peroxide influenced the 24 hr. phage count of lysogenic strains. This could be accounted for to some extent by the inactivation of the hydrogen peroxide by catalase, which was produced by all strains of *Ps. pyocyanea.* In the case of F1 acting on culture C10, the hydrogen peroxide would inactivate the free phage before the production of catalase by the host had commenced. With lysogenic strains, if the phage initially within the host cell were unaffected by the hydrogen peroxide, when the latter had been inactivated by catalase, the bacteriophage could reproduce normally. For similar reasons, together with the fact that hydrogen peroxide has no action in broth, notatin would be inactive in the agar plate test even in the presence of glucose.

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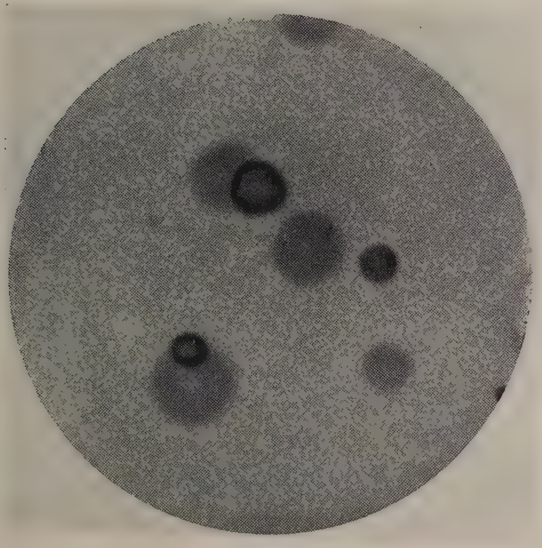

L. Dickinson—The bacteriophages of *Pseudomonas pyocyanea*. Plate 1

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EXPLANATION OF PLATE

Ps. pyocyanea bacteriophage plaques (×5); showing the larger Pa-type with diffuse halo and the small, clear Pb-type.

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Some Factors Affecting the Growth and Sporulation of *Chaetomium globosum* and *Memnoniella echinata*

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SUMMARY: The influence of certain carbohydrates, nitrogen compounds and accessory substances on the growth and sporulation of *Chaetomium globosum* and *Memnoniella echinata* has been studied. It has been confirmed that *M. echinata* needs an external supply of biotin, which has also a slight effect on the growth of *Chaetomium globosum*. For *C. globosum*, a very low level of soluble sugar in the medium was essential for the production of perithecia. With adequate biotin, *Memnoniella echinata* sporulated in the presence of considerable concentrations of sugar, but at low biotin levels no sporulation occurred until soluble sugar approached exhaustion.

Jute extract stimulated growth and accelerated sporulation of *Chaetomium globosum*; this was not due to the presence in the extract of any of nine well-known B-group vitamins. Jute extract had no more influence on *Memnoniella echinata* than would be due to its biotin content.

In the course of an extensive survey of the cellulose-decomposing capacity of certain mildew-fungi, which commonly occur on textiles, it was noticed that sporulation in some cases was markedly influenced by the nature of the culture medium employed. Some *Chaetomium* species, for instance, gave only sterile hyphal growth on ordinary salt-sugar media, whereas in the presence of cellulose vigorous perithecial fruiting was observed. Various factors, physical and chemical, have been reported by previous workers as influencing sporulation in a number of species, and the present studies were undertaken chiefly in order to gain more information concerning the conditions needed to stimulate fruiting of *C. globosum* Kunze ex Fr. and *Memnoniella echinata* (Rw.) Galloway.

EXPERIMENTAL

Materials and methods

Chaetomium globosum. The strain used was originally isolated from a piece of brown paper found rotting in contact with moist soil, and was maintained on a strip of filter-paper half immersed in Czapek-Dox salt solution.

Memnoniella echinata. The strain was isolated in India from rotten cotton canvas, and was maintained in the same way as the *Chaetomium* sp. The identity of both organisms was confirmed by the Imperial Mycological Institute, Kew.

The basal medium contained $NaNO_3$ 2 g., KH_2PO_4 1 g., KCl 0·5 g., $MgSO_4.7H_2O$ 0·5 g., and $FeSO_4.7H_2O$ 0·01 g., in 1 l. water. Occasionally, as indicated in the text, K_2HPO_4 replaced the acid phosphate. For solid media, agar (15 g./l.) was incorporated. Sterilization was at 10 lb. for 15 min.; incubation was at 30°.

In the tables, the symbols + to + + + have been used to indicate the amount of growth and sporulation; attempts were made to count the perithecia of *C. globosum*, but had to be abandoned as the hyphal growth often obscured the perithecia. Colony diameters were used in some cases to estimate growth on agar media, two measurements at right angles being taken on pairs of plates; in liquid media, the dry weight of mycelium was determined.

Source of carbon in the medium

A low concentration of hexose sugar has frequently been associated with production of spores (cf. Klebs, 1900; Coons, 1916; Robinson, 1926), and any process resulting in exhaustion of sugar in the medium (e.g. prolonged incubation, accelerated respiration, etc.) may finally cause sporulation. The nature of the carbohydrate present has also been thought to affect sporulation, but in some cases this effect seems to have been due merely to the ease with which a polysaccharide is broken down to hexose. In the first tests, which were mainly qualitative, eight different carbohydrates, chosen to represent a range of chemical types, were used as the source of carbon, namely, glucose, maltose, cellobiose, sucrose, starch-dextrin, cello-dextrin, starch and cellulose. All were used in 3 % concentration, except cellulose (a sample purified by refluxing with 1 % ethanolic HCl, followed by 1 % NaOH and washing), whose concentration was 1 %; the basal medium contained K_2HPO_4 in place of the acid salt. Table 1 records the growth after 14 days' incubation.

Table 1. *Influence of various carbohydrates on growth and sporulation on solid media*

	Glucose	Maltose	Cellobiose	Sucrose	Starch-dextrin	Cello-dextrin	Starch	Cellulose
				Chaetomium globosum				
Mycelial	++	++	++	++	++	+	++	+
Perithecial	–	Trace	–	–	Trace	+++	++	+++
				Memnoniella echinata				
Mycelial	++	++	++	++	++	++	++	+
Conidial	–	Trace	–	–	++	+++	++	++

The effect of varying the sugar concentration was next studied, the results observed after 10 days being recorded in Table 2, whereas Table 3 gives the results of a more detailed study of growth on glucose media, with varying concentrations and over longer periods.

Because sporulation seemed connected with a low concentration of sugar in the medium, whether initially provided or produced by prolonged incubation, the actual sugar concentration at the time of onset of fruiting was determined in some cases. The results (Table 4) showed that with *C. globosum* fruiting did not on any occasion take place until the sugar had almost disappeared from the medium; nevertheless, exhaustion of the sugar did not necessarily induce fruiting, and some other factor must therefore have played a part in the process. With *Memnoniella echinata* exhaustion of sugar is not essential, for

Table 2. *Effect of varied concentration of sugars on growth and sporulation on solid media*

Chaetomium globosum

	Sugar (%)											
	0·1		0·5		1·0		1·5		2·0		2·5	
	m.	p.	m.	p.	m.	p.	m.	p.	m.	p.	m.	p.
Glucose	+	++	++	++	+++	−	+++	−	+++	−	+++	−
Maltose	+	++	++	++	(Not examined)							
Sucrose	+	++	++	++	+++	−	+++	−	+++	−	+++	−
Cellobiose	+	++	++	++	(Not examined)							

Memnoniella echinata

	Sugar (%)							
	0·1		0·5		1·0		1·5	
	m.	c.	m.	c.	m.	c.	m.	c.
Glucose	+	+	++	Trace	++	−	++	−
Maltose	+		++	−	++	−	++	−

m., p. and c. = mycelial, perithecial and conidial growth.

Table 3. *Effect of different concentrations of glucose on growth and sporulation, over varying periods of time, of* Chaetomium globosum *on solid media*

	Days							
	10		15		20		25	
Glucose (%)	m.	p.	m.	p.	m.	p.	m.	p.
0·1	+	++	+	++	+	++	+	++
0·5	++	+++	++	+++	++	+++	++	+++
1·0	++	−	++	+++	++	+++	++	+++
1·5	++	−	++	−	++	++	++	+++
2·0	+++	−	+++	−	+++	−	+++	++

m. and p. = mycelial and perithecial growth.

when the initial glucose level was high, considerable amounts of sugar remained at the time of sporulation (Table 4). However, the medium for *M. echinata*, unlike that for *Chaetomium globosum*, contained added biotin, which tests had shown to be an essential factor for growth in liquid media (see p. 165).

Source of nitrogen in the medium

The influence of different types of nitrogen compounds on the growth and sporulation of *C. globosum* was investigated, in the presence of glucose (0·5 %), as the source of carbon. The substances used were sodium nitrate, ammonium sulphate, asparagin (twice recrystallized) and casein hydrolysate (Ashe 'vitamin-free'), the nitrogen concentration being in each case 0·033 %. The rate of growth of the organism was estimated by measuring colony-diameters at intervals (Table 5).

Growth factors

It was found that the *C. globosum* and the *Memnoniella echinata* differed in their response to some of the recognized members of the B-group of vitamins, and it will be more convenient to consider each organism separately.

Table 4. *Initial concentration of glucose, concentration at time of sporulation, and time for sporulation in liquid media*

Chaetomium globosum

Glucose (%)		
Initial	At onset of sporulation, or after 44 days	Days required for sporulation
0·10	< 0·015	8
0·30	< 0·015	18
0·50	< 0·015	> 44
2·00	< 0·015	> 44

Memnoniella echinata

Glucose (%)		
Initial	At onset of sporulation	Days required for sporulation
0·50	0·16	5
1·00	0·65	5
1·50	1·30	5

Table 5. *Influence of various sources of nitrogen on colony diameter (mm.) of* Chaetomium globosum *on solid media*

	Days									
	1	3	4	5	6	7	8	10	11	13
Sodium nitrate	1·5	8	11	13·5	16·5	20	23	24*	25	28
Ammonium sulphate	0	3	3·5	4	4·5	4·5	4·5	4·5	4·5	4·5
Asparagin	1·5	8	10	12	14	16·5	19	27·5	32	38*
Casein hydrolysate	1·5	13·5	16·5	20	22	24*	26·5	32	37	43

* First appearance of perithecia.

M. echinata. The essential nature of biotin for this organism, established by Marsh & Bollenbacher (1946) was confirmed in the present work. On liquid media containing 3% (w/v) glucose, growth was insignificant, but the addition of as little as 0·002 μg. biotin/20 ml. medium produced a noticeable response, and more than 500 times this amount seemed to be required for saturation at this concentration of glucose (Table 6).

Table 6. *Effect of biotin on germination and growth of* Memnoniella echinata

	Biotin (μg./20 ml. medium)				
	0·0002	0·002	0·01	0·02	0·1
Germination period (days)	3	2	2	1	1
Dry weight of mycelium after 10 days (mg.)	6	19	76	113	154

Among other recognized members of the vitamin B group. thiamin, pyridoxin, nicotinic acid, riboflavin, pantothenic acid and *p*-aminobenzoic acid were without activity, and it appears that biotin represents the only B vitamin requirement of *M. echinata.* It may be mentioned that growth on agar media, without added biotin or at very low biotin levels, was always better than on corresponding liquid media, suggesting the presence of a trace of biotin in the agar (cf. Table 2). The nature of the mycelium was markedly different at different biotin concentrations; in liquid media containing 0·01 μg. % or less, white detached colonies were formed, whereas in greater concentrations of biotin brownish gelatinous mats appeared. On agar media the type of growth was thin and spreading ('starvation' type) at low biotin concentrations.

Chaetomium globosum. During some experiments in which an aqueous extract of jute was used as a source of nitrogen, a remarkable stimulation of growth and formation of perithecia in *C. globosum* was noticed; and there was very ready fruiting when the organism was grown on jute fibres suspended in salt solutions. These facts suggested the intervention of some factor of the vitamin type, and led to experiments designed to test this supposition.

The extract was prepared from a 'white' jute of recent origin (*Corchorus capsularis*; grown at Kishoreganj, 1945) as follows. Five g. of the clean dry fibres were boiled for 30 min. with about 100 ml. of distilled water, after which the extract was squeezed out and the fibre discarded. The liquor was evaporated on the water-bath to less than 50 ml. and filtered to remove a dark scum which formed during evaporation. The filtrate was added to 50 ml. of double strength basal salt medium, glucose and agar were added as required, and the whole made up to 100 ml. Thus 100 ml. of the final medium contained the extract from 5 g. of jute, the actual weight of solid incorporated being about 50 mg. The effect of the jute extract on growth and fruiting, in a medium containing 1 % glucose and 1·5 % agar, and the results of comparative tests in which Marmite and malt extract (each at 1 % w/v) were used as sources of growth factors, are shown in Table 7. In all cases growth was markedly stimulated, especially with Marmite, but fruiting was observed at a much earlier stage with jute extract, and the final crop of perithecia was very much greater. It may be pointed out also that Marmite and malt extract contributed an appreciable amount of solid matter to the medium, the jute extract much less.

The effect of pure vitamins was next studied, when it was found that thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid and *p*-aminobenzoic acid, in concentrations of 10, 25 and 50 μg./100 ml. of medium, and folic acid and inositol in concentrations of 5 μg. and 10 mg./100 ml. respectively, had no significant effect on growth or fruiting. Biotin appeared to accelerate fruiting to a slight extent, but the effect was in no way comparable with that of the jute extract (Table 8).

An attempt was made to estimate the quantities of known growth substances in jute extract, with a view to simulating the effect of the extract with an artificial mixture in the correct proportions. Assays were made by the methods described by Barton-Wright (1946), except that thiamin was estimated by the method of Bonner & Erickson (1938). On the basis of these results (Table 9), the pure vitamins corresponding to 5 g. of jute were incorporated into 100 ml. of basal agar medium, containing 0·5 % glucose.

This mixture, though possibly causing a slight acceleration of fruiting, was in no way as active as the crude extract from jute (Table 10).

Table 7. *Effect of Marmite, malt extract and jute extract on growth and sporulation of* Chaetomium globosum

(*a*) Colony diameters (mm.) on agar media

	Days								Perithecia at 14 days
	1	2	3	4	5	6	7	10	
Marmite	2·5	12	13*	28	30	31	32·5	46	++
Malt extract	3	10	10*	25	27	27·5	28	34·5	+ (Immature)
Jute extract	3·5	16	33*	40	47	52	55·5	60	+++

* First appearance of perithecia.

(*b*) Dry weight of mycelium (mg.) in liquid media

	Days		Days required for sporulation	Perithecia at 14 days
	3	6		
Marmite	62, 64	131, 134	8	+
Malt extract	35, 31	61, 62	8	+
Jute extract	52, 54	90. 94	6	+++

Table 8. *Effect of biotin on sporulation of* Chaetomium globosum *on solid media*

Glucose (%)	Biotin (μg. %)	Day of fruiting
0·5	0	8–10
	4	6–9
1·0	0	11–12
	4	9–11
1·5	0	15
	4	10–12

Table 9. *Assay of vitamins in jute extract*

Vitamin	Assay organism	Mode of computation	μg. vitamin/g. jute
Thiamin	*Phycomyces blakesleeanus*	Direct reading	0·222
Riboflavin	*Lactobacillus helveticus*	Slope-ratio	0·301
Pyridoxin	*Neurospora sitophila*	Slope-ratio	0·116
Nicotinic acid	*Lactobacillus arabinosus*	Direct reading	0·471
Pantothenic acid	*Lactobacillus arabinosus*	Direct reading	0·259
Biotin	*Lactobacillus arabinosus*	Direct reading	0·0089

Estimations of residual sugar at the time of fruiting again showed that exhaustion of soluble sugar was in some way connected with sporulation; at all concentrations of glucose, fruiting was much accelerated, and this was especially so when the initial glucose was 2 % or more, as previous experiments, using media without added growth factors, had failed to give fruiting even after 44 days (Table 11, cf. Table 4).

The rate of glucose utilization in media containing initially 0·25 % of the sugar was determined in presence and in absence of jute extract. The results (Fig. 1) indicated that sugar consumed per unit weight of mycelium remained fairly constant throughout the entire growth period, and was the same on both media. In each case the rate of consumption increased till the end, and fruiting was preceded by the period of most rapid utilization. Jute extract, by stimulating growth, greatly increased the rate and perithecia appeared sooner.

Table 10. *Comparison of the effect of jute extract and mixed vitamins on colony diameters (mm.) of* Chaetomium globosum *(solid media)*

	Days									
	1	2	3	5	6	7	8	9	10	12
Basal medium	0	3·5	6·5	13	17	20·5	23	25*	27	28·5
Mixed vitamins	0	4	6·5	11·5	14	19	22*	25	27	30
Jute extract	3	15	26*	44	51	57	64	69	75	—

* First appearance of perithecia.

Table 11. Chaetomium globosum *in liquid media; concentration of glucose at time of sporulation in presence of jute extract*

Glucose (%)		Days required for fruiting
Initial	At onset of fruiting	
0·10	<0·015	5
0·30	<0·015	5
0·50	<0·015	7
1·00	<0·015	11
2·00	<0·015	22

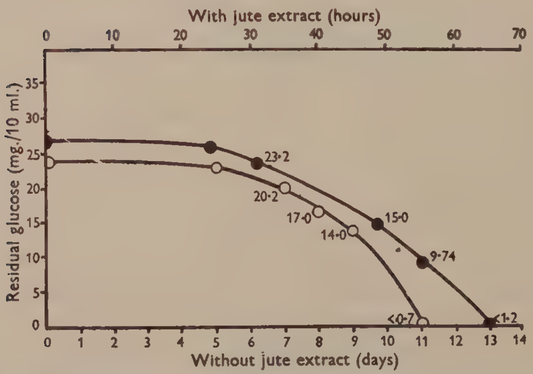

Fig. 1. The rate of sugar consumption by *Chaetomium globosum* in liquid media. ●—● in presence of jute extract, ○—○ in absence of jute extract.

These observations suggested that fruiting was stimulated by the action of some definite chemical substance, present in notable amounts in jute, but not any one of the six vitamins assayed or of the other three tested. Such a substance might be slowly produced by the organism itself, attainment of a critical concentration being necessary for fruiting. If such were the case, an extract of a vigorously fruiting culture might stimulate sporulation in a vegetative culture, or it might be possible to detect such a substance in the surrounding medium. To test this, *Chaetomium globosum* was grown in 200 ml. of liquid medium containing 0·1 % of glucose; the mycelium was harvested on the tenth day, when fruiting was vigorous, and an extract was made by grinding up the mycelium with 40 ml. of fresh basal medium. This extract was filtered and incorporated in a fresh batch of medium, with 0·5 % glucose. Similarly, the staled liquid medium was adjusted to its original pH (5·8) and the glucose concentration brought to 0·5 %. In each case 1·5 % agar was added, and after sterilization fresh inoculations were made. Table 12 shows the relative growth and fruiting properties of the original medium and those incorporating mycelial extract or metabolic products.

Table 12. *Effect of mycelial extract and staled medium on colony diameters (mm.) of* Chaetomium globosum *on solid media*

	Days							
	1	2	3	4	7	8	9	10
Basal medium	0	3·5	6·5	—	20·5	23	25*	27
Mycelial extract	3·5	7·0	10·5	14	26*	31	34	35
Staled medium	2·5	6·5	8·5	13	25*	27	31	34

* First appearance of perithecia.

Influence of contaminant fungi

The mutual stimulation of organisms growing in association is well known and can usually be ascribed to the production by each of the species of a growth factor essential for the other (cf. Kögl & Fries, 1937). Tests were accordingly made to discover whether other fungi could stimulate fruiting in *C. globosum* or *Memnoniella echinata* grown on agar media containing 0·5 % glucose. Fifteen species, all of which except the *Memnoniella* sp. grew and sporulated well on defined media, were tried on *Chaetomium globosum*; these included four species of *Aspergillus*, five of *Penicillium*, one each of *Trichoderma*, *Paecilomyces*, *Chaetomium*, *Stachybotrys* and *Memnoniella*, and another *Dematiaceae* species. The test fungus was planted 2 days before the *Chaetomium globosum*. In no case was any stimulation of fruiting observed, and perithecia when they appeared always began to develop from the centre of the colony, and not at the edge nearest the contaminant organism. With *Memnoniella echinata*, on the other hand, the presence of any one of a wide variety of fungi induced fruiting at the edge nearest to the foreign colony.

DISCUSSION

From previous work, as well as from that recorded here, it is evident that many factors, both chemical and physical, may affect the processes whereby the mycelial habit of growth gives place to the sporulating. Among other factors must be counted: (*a*) the degree of 'maturity' of the organism; (*b*) the general nutritional level, especially perhaps as regards carbohydrate supply; and (*c*) the possible action of certain stimulants necessary in minute amounts and thus resembling (or in some cases perhaps identical with) the long recognized growth factors.

That the organism must attain a certain state of development before fruiting can occur seems to be unquestionable; for instance, on a 0·5 % glucose medium, *Chaetomium globosum* never produced perithecia until the colonies had reached a diameter of some 20 mm. irrespective of whether this occurred in 3 or in 10 days. The suggestion that some substance formed by the organism itself must reach a critical concentration to induce fruiting receives some support from the observation that fruiting is accelerated by extracts of the mature organism.

The experiments on sugar utilization indicate that the general nutritional level, especially as regards carbohydrates, is implicated; for with *C. globosum* in liquid media, fruiting was in every case delayed till the free sugar in the medium had been reduced to a very low concentration, either owing to almost complete utilization, or because soluble sugar was available only at low concentrations as a result of its slow liberation from a polysaccharide. Previous workers have recorded that media of low sugar content favour fruiting in many fungi (cf. Hawker & Chaudhury, 1946). The apparent anomaly (Tables 7(*a*) and 9) that the organism continues to grow in agar media after the onset of sporulation, and may more than treble the diameter of colonies, is explained by the slow diffusion of sugar in the agar; the perithecia form in the centre of the colony, where sugar approaches exhaustion, while fresh growth takes place at the periphery.

It is clearly possible that certain chemical substances may be necessary for the initiation of sporulation. The influence of thiamin and biotin on fruiting in various species has been made evident during the past decade, and the effect of biotin on *Memnoniella echinata* again appears here. Among the carbohydrates examined, it is perhaps significant that those which at first sight seemed to be associated with rapid production of perithecia were all colloidal substances of high molecular weight, prepared from plant material, upon which traces of growth substances from the plant tissue might well remain adsorbed. Maltose, an apparent exception to this, has been shown by Schopfer (1932, 1934) to be liable to contamination with thiamin.

Increased growth was frequently found to result from the presence of various 'natural' extracts (malt, yeast, etc.) in the media, and in some cases an appreciable acceleration of fruiting was also observed; even an extract of agar gave a small response in liquid media. The presence of members of the B-group may well have accounted for the increased growth, but none except perhaps biotin directly affects sporulation. Of all the materials tested, the extract of jute is outstanding in stimulating the formation of perithecia in *Chaetomium globosum*, not only in the shortest time, but also in the greatest quantity, and suggests the existence of a definite 'fruiting factor' other than the recognized B-group vitamins. Only biotin, of all the known substances tested, had any detectable effect on the fruiting of *C. globosum*. This substance is almost certainly produced and liberated by many fungi, as shown by the stimulatory effect of many species grown in association with *Memnoniella echinata*, for which it is the only essential growth factor; yet none of the species which were found to stimulate *M. echinata* had the least effect on *Chaetomium globosum*. The factor in jute is therefore not biotin, nor any other of the nine B-vitamins tested.

Such a substance may accelerate sporulation merely by causing a more rapid utilization of sugar, for instance, by stimulating respiration (since exhaustion of sugar seems a prerequisite of fruiting in *Chaetomium*); or it may act in a more specific and direct manner, that is, by taking part in some enzyme system immediately concerned in the differentiation of the fruit bodies. That such factors are confined to jute is by no means suggested. Robbins & Ma (1942) found in cotton not only biotin and pyridoxin, but also unknown factors 'favourable to *Ceratostomella*', while Zuck & Diehl (1946) demonstrated that certain other species grew and fruited on cotton-extract media, but not on defined media (see also Sherwood & Singer, 1944). A small amount of some stimulant apparently occurs in agar, and Tschudy's (1937) finding that *Chaetomium* species fruited better on agar media than in liquid would seem due to such factors, and not to the action of nutrients such as pentosans, as he suggests. The observation that extracts of mature mycelia, or of staled media, accelerate fruiting not only indicates that a definite chemical influence is at work, but is again in agreement with the suggestion that the 'fruiting factor' may be distinct from the recognized growth factors.

Memnoniella echinata differs in some important respects from *Chaetomium globosum*. In the first place, biotin is essential for growth. In its presence in adequate amounts, exhaustion of sugar is not an essential prerequisite for sporulation; nevertheless, with very low levels of biotin, sporulation is connected with low levels of soluble sugar. If fruiting is initiated directly by a chemical stimulant other than biotin, then this must be produced in sufficient quantities by the organism itself; no indication of any other external factor was detected, and even the jute extract, so active for *C. globosum*, had no more effect than could be ascribed to its biotin content. One anomaly remains to be mentioned. The method of preparation of cello-dextrin, involving fairly drastic chemical processes (e.g. acetolysis), was expected to destroy any biotin present in the original cellulose; nevertheless, cello-dextrin distinctly stimulated growth and sporulation, and the possibility still remains that some at least of the high-molecular polysaccharides may have their own influence on metabolism.

The authors wish to record their thanks to the Committee of the Indian Jute Millers' Association Research Institute, by whom the work was supported, and to Dr S. E. Jacobs and Dr L. E. Hawker, for helpful discussion.

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ADDENDUM

Note on the biotin requirements of Stachybotrys atra

A culture of *Stachybotrys atra* (kindly supplied by Mr L. D. Galloway), while giving only a poor mycelial growth on a liquid medium containing 3 % glucose, was found to grow well and to produce abundant conidia in agar media, and in liquid media when the glucose was replaced by filter-paper. Marsh & Bollenbacher (1946) reported a partial deficiency of biotin in this organism, and the above observations are in agreement with this view.

EXPLANATION OF PLATE

Chaetomium globosum grown on agar containing inorganic salts and 1 % glucose. Petri dishes 7·5 cm. diameter.

Fig. 1. Growth and sporulation after 6 days. Left to right, the basal medium alone, with 1 % malt extract, and with extract of 5 g. jute/100 ml.

Fig. 2. Growth and sporulation after 14 days. Left to right, basal medium with 1 % malt extract, with 1 % marmite, and with extract of 5 g. jute/100 ml.

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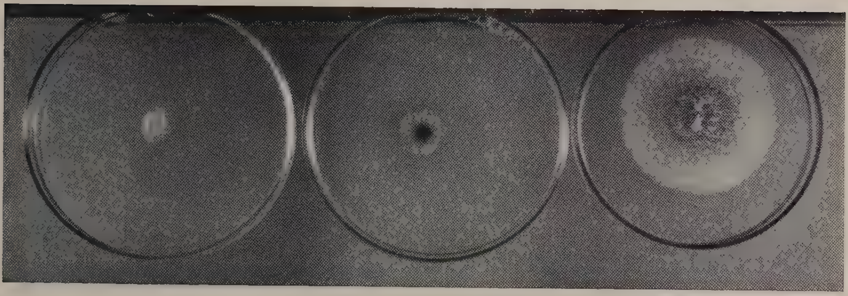

Fig. 1

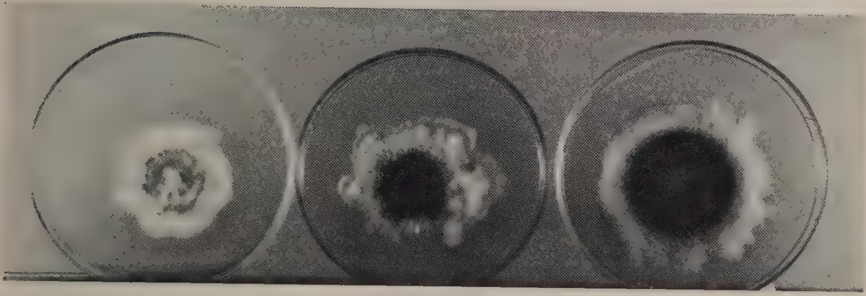

Fig. 2

H. W. Buston and S. N. Basu—Some factors affecting the growth and sporulation of *Chaetomium globosum* and *Memnoniella echinata*. Plate 1

Variations in the Properties of Potato Virus *X* and their Effects on its Interactions with Ribonuclease and Proteolytic Enzymes

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SUMMARY: When concentrated by precipitation with acid and salts, or by high-speed centrifugation, potato virus *X* tends to become insoluble though still remaining infective and serologically active. This greatly complicates purification and no method was found that could be relied upon to give good yields of virus with constant properties. Insolubility is correlated with the aggregation of virus particles to form long threads that become entangled, but it is probable that combination of the particles with some cell constituents is also concerned.

Insoluble preparations dissolve slowly when incubated with pH 7·5 borate buffer, and rapidly in the presence of trypsin or chymotrypsin. Both of these enzymes hydrolyse virus *X*, chymotrypsin being the more effective, but different strains of the virus vary in their susceptibility.

Ribonuclease readily hydrolyses the nucleic acid derived from virus *X*, but seems to have no enzymic action on the active virus. When mixed with the virus, the enzyme combines with it and reversibly inhibits infectivity. At pH 7 the addition of ribonuclease to soluble virus preparations causes loss of anisotropy of flow, a fall in precipitin titre, and the production of an insoluble complex. Incubation at pH 7·5 with borate buffer slowly dissolves the complex and restores the original properties of the virus; the rate of re-solution is increased by the presence of trypsin. Some preparations of the virus were partially decomposed by incubation with borate buffer, and sometimes the rate of decomposition was increased in the presence of ribonuclease.

Of the plant viruses that have been isolated in the form of nucleoproteins, potato virus *X* is exceptional in being susceptible to the proteolytic enzymes trypsin, pepsin and papain (Bawden & Pirie, 1936, 1938). Tobacco mosaic virus, which resembles virus *X* in forming rod-like particles of variable lengths and contains the same proportion of nucleic acid to protein, is hydrolysed by these enzymes only if it is first denatured, though trypsin combines with it and inhibits its infectivity (Bawden & Pirie, 1937; Kleczkowski, 1944). Similarly, ribonuclease combines with tobacco mosaic virus, and is an even more powerful inhibitor of infectivity than trypsin, but it does not hydrolyse the nucleic acid unless the virus is first denatured (Loring, 1942). It seemed of some interest, therefore, to determine the effects of ribonuclease on virus *X*; the work described below was started for this purpose, but for various reasons the problem proved unexpectedly complex. There was little difficulty in demonstrating that any enzymic activity ribonuclease may have on virus *X* is small compared with that of the proteolytic enzymes, but it was less easy to find with certainty whether or not it has any such activity. The enzyme inhibits infectivity and its interactions with the virus also affect serological reactions, so that quantitative interpretations of infectivity and precipitin tests are uncertain. By far the greatest complication was the irregular behaviour of the virus during the course of purification, and its tendency to become insoluble, a phenomenon not encountered with tobacco mosaic or other viruses that have been purified. No method of purification was found that could be relied on to give preparations with reproducible properties, and although consistent results were obtained in repeated tests on the same preparation, these might not be reproduced on other preparations. The work was therefore extended to study some of these variations in the behaviour of virus X during purification and their effect in determining the interactions between the virus and enzymes.

MATERIALS AND METHODS

Three strains of potato virus X were used, namely, X^K, X^S and X^Y. Most of the work was done with tomato (*Lycopersicum esculentum*, var. Kondine Red) and tobacco (*Nicotiana tabacum*, var. White Burley) as host plants, but some tests were also made with X^K and X^Y propagated in *N. glutinosa*, *Datura stramonium* and potato (*Solanum tuberosum*, vars. Majestic and Doon Star). In tomato and potato plants X^K and X^S cause a severe mosaic with much necrotic spotting and deformity of the leaves; in tobacco, *Nicotiana glutinosa* and *Datura stramonium*. they cause necrotic local lesions and systemic symptoms of the ringspot type. In all the hosts, X^Y causes a bright yellow interveinal mosaic with little or no necrosis. However, the precise symptoms caused by any of the strains vary considerably with the season and with the age and condition of the host plants at the time of inoculation. For producing large quantities of virus X, tomato is the most suitable host, as young plants produce much sap which has a virus content twice as great as that from tobacco or potato. On the other hand, tobacco has the advantage that purification of the virus usually proceeds more smoothly. In the various hosts used, strains X^K and X^Y occur at approximately twice the concentration of X^S. The virus content of sap is higher from plants raised during the winter than during the summer, and purification is also easier with sap from the former. Sap from tomato plants, infected with X^K and X^Y, has sometimes during the winter reached a virus content of 2 g./l. Leaves are picked from plants about a month after infection, when they are showing good symptoms. After removing the petioles and the largest parts of the main veins, the laminae are minced by passage through a domestic meat mincer, and the sap which is expressed by squeezing the minced leaves in a cloth bag forms the starting material for purification.

Infectivity tests were made using strains X^K and X^S by the local lesion method with tobacco or *Nicotiana glutinosa* as a test plant. Precipitin tests were made by methods previously described (Bawden & Pirie, 1936), 1 ml. of antiserum at a constant dilution being added to a series of tubes containing 1 ml. of virus preparation at different dilutions. The greatest dilution to give a precipitate visible to the eye after 3 hr. incubation in a water-bath at 50° was taken as the precipitin titre. Anisotropy of flow was observed merely by examining the fluids in polarized light as they are tipped from end to end of half-filled tubes 0·5 cm. in diameter.

Measurements of enzymic activities were made by methods previously described (Kleczkowski, 1944, 1946). Crystalline chymotrypsin was obtained from beef pancreas by the method described by Northrop (1939). After isolation and recrystallization as chymotrypsinogen, it was converted into chymotrypsin and recrystallized twice. Ribonuclease was isolated from beef pancreas, and was crystallized from ammonium sulphate solution by the method described by Kunitz (1940). In this state, however, preparations were unsuitable for use with virus *X*, as they contained sufficient proteolytic activity to hydrolyse the virus. Hence, before use, the preparations were heated as described by Kleczkowski (1948), to destroy proteolytic activity but leaving much of the ribonuclease activity unimpaired.

Bacteria readily attack potato virus *X*, which is also destroyed by incubation with phosphate buffer around pH 7 (Bawden & Crook, 1947). All incubations were therefore made with solutions containing 0·05–0·1 M boric acid borax buffer, which has sufficient antiseptic properties to prevent bacterial growth and is relatively innocuous towards the virus.

Variation in the properties of virus during purification

In attempts to find a method of purification that would give consistent results, various methods of clarifying the sap were tried, both singly and in combination. These were the addition of Na_2HPO_4 (10 g./l.), freezing and thawing, and heating to 60° before centrifuging for 15 min. at 3000 r.p.m. All of them produced clear brown supernatant fluids, and seemed to cause little loss of virus on the green sediments, but even the combination of all three failed to leave virus that would behave consistently. At this stage there are considerable differences between different lots of sap, indicating the wide range of virus concentration produced by the different strains in different host plants. Some lots of sap, especially those from tomato plants infected with X^Y or X^K in the winter, show anisotropy of flow strongly and precipitate with virus antiserum when diluted as much as 1/5000. Others, for example, sap from tobacco plants infected with X^S in the summer, show no anisotropy of flow and give precipitin titres of only 1/250. There is, however, no correlation between virus concentration of the sap and behaviour during subsequent purification treatments: better preparations of virus can sometimes be made from samples of sap with low than with high virus contents, but not regularly.

The main difficulties in purifying virus *X* are its tendency to become insoluble and the readiness with which it adsorbs other materials. When they first described a method for making liquid crystalline preparations, Bawden & Pirie (1938) commented on the greater difficulty of purifying virus *X* than tobacco mosaic virus and suggested that their yields were probably only about one-fifth of the virus originally present in the sap. A treatment they found most valuable for obtaining consistent products was incubation with trypsin, but this destroyed some virus. As the main purpose of this work was to obtain virus *X* in a form suitable for testing its susceptibility to ribonuclease, this treatment was inadmissible for fear that some residual trypsin might complicate subsequent tests with ribonuclease. The omission of this treatment is probably responsible for most of the difficulties encountered, and the attempt to substitute sedimentation in the ultracentrifuge as an alternative was far from successful. Any treatment that precipitates the virus, at any stage during the course of purification, is likely to cause a part or the whole of the virus to become insoluble. A precipitation procedure that may have been applied several times previously without rendering a significant fraction of the preparation insoluble, when repeated, may turn the major part or even the whole of the preparation insoluble. Bawden & Pirie (1938) found that the brown precipitates discarded during the course of preparations contained some virus, but in our work the bulk of the virus has often been contained in insoluble pigmented material. There is little in the appearance and behaviour of such materials to suggest this, and they could easily be regarded as impurities whose removal would produce a useful fractionation.

No exact sequence or number of fractionations can be described for the purification of virus *X* because the behaviour of different lots of sap is so variable that each has to be treated on its merits, but we have used various combinations of fractional precipitation and differential ultracentrifugation. All the virus can be precipitated from sap by quarter-saturation with $(NH_4)_2SO_4$ and it can usually be dissolved completely in a volume of water equal to a fifth of that of the original sap. Provided the fluids are kept neutral by the addition of NaOH, precipitation and re-solution in water can be repeated many times without serious loss of virus, though occasionally, even at this early stage of preparation, the virus contained in the bulky chocolate-coloured precipitates produced by $(NH_4)_2SO_4$ dissolves incompletely or only slowly.

Each time a precipitate is suspended in water and centrifuged to separate insoluble material it is advisable to examine the supernatant fluids for anisotropy of flow. This provides a rapid test for the presence of the virus and shows whether most has dissolved or has remained on the precipitate. When the supernatant fluids from the ammonium sulphate precipitations are colourless, the redissolved precipitate is brought to pH 4·5 with HCl. This produces a variable result, sometimes precipitating all the virus, most often the greater part, but occasionally little or none. The differences are not correlated with virus strains or host plant, but different proportions of different preparations precipitate at different pH values. If a precipitate obtained at pH 4·5 is taken up at pH 7 and the fluid again adjusted to 4·5, it is usual for only a part of the preparation to precipitate. The treatment with acid, however, is useful, because it often denatures some contaminants that are not removed by the treatment with ammonium sulphate. The precipitate is taken up at pH 7; if much virus remains in the supernatant fluid at pH 4·5 this is neutralized, mixed with the virus that precipitated, and the mixture centrifuged to free from any insoluble material. Additional precipitations with $(NH_4)_2SO_4$ and re-solutions now usually remove further coloured contaminants, and it is also useful to sediment the virus by centrifuging 1 hr. at 30,000 r.p.m. However, any attempt to concentrate the virus to solutions containing more than about 5 mg./ml. is likely to lead to the separation of the virus in insoluble, or very slowly dissolving states. Often the pellets obtained by ultracentrifugation need extracting several times, using a total volume of water equal to that of the originally centrifuged fluid, before they resuspend completely and remain stable when centrifuged at 3000 r.p.m. The virus is more stable if suspended in 0·1 M borate buffer, pH 7, than in water, and preparations that have remained stable during the course of several precipitations or ultracentrifugations may become wholly insoluble when dialysed against distilled water.

Aggregation and disaggregation of virus particles

The difficulty experienced in keeping virus *X* in solution partly arises because, during purification and concentration, the virus particles aggregate linearly to such an extent that they become entangled one with another and form a mesh-like structure too bulky to give a stable suspension or solution. This lengthening and entanglement of the particles is clearly shown by electron micrograms of the virus preparations at different stages. In sap the virus occurs as separate particles varying greatly in length, but most are less than 500 $m\mu$. long. In the insoluble preparations, on the other hand, individual particles are impossible to identify, and the long fibres are so intertwined that electron micrograms resemble pictures of fishing nets. In this condition the virus also ceases to show anisotropy of flow, or shows it much less than when the virus is in solution and present as individual rods. Insolubility, however, does not seem to result simply from the aggregation of virus particles, but is more probably also associated with the combination of the virus with some other materials. The fibres composing the insoluble preparations are coarser than the virus particles occurring in sap or than the elongated threads in purified soluble preparations. In addition, the mesh-like structure appears to contain particles of a different shape and denser material than the virus.

Bawden & Pirie (1945) have shown that small particles of tobacco mosaic virus aggregate linearly when incubated with trypsin, and they suggest that the trypsin removes materials combined with the ends of the particles and sets free groups of the particles that then combine with one another. With this virus, aggregation does not proceed sufficiently far for the virus to come out of solution, or to sediment with low-speed centrifugation. Bawden & Crook (1947) have described a similar aggregation of small particles of virus *X* produced by short incubations with trypsin. It is possible, therefore, that virus *X* particles as they originally occur in the sap are combined with materials that act as stabilizers, and whose removal during purification causes the particles to become increasingly aggregated, but this does not explain all the phenomena. For example, the degree of aggregation should be correlated with the purity of a preparation, as it is with tobacco mosaic virus, but this is not always so. Dark brown precipitates, which will not dissolve in water, often separate during the early stages of preparing virus *X*, and these are rich in highly aggregated virus. It is likely that in these conditions the virus has formed an insoluble complex by combining with some other constituent of sap. The insoluble virus seems to be fully infective and is serologically active, but suspensions show no anisotropy of flow. Incubation with trypsin restores anisotropy of flow and changes the character of electron micrograms from the fishing-net picture to one of more discrete, though still greatly elongated, particles. After incubating such precipitates at 37° for 30 min. with 0·5 % commercial trypsin, about 70 % of their weight can be recovered in the form of soluble virus showing strong anisotropy of flow. The virus in such precipitates can also be brought into solution slowly by incubation at 37° with pH 7·5 borate buffer, though 3 days' incubation may be needed to produce the same result as 30 min. with trypsin (Table 1).

Table 1. *The effect of trypsin on a crude insoluble preparation of virus* X^K

1·8 % virus suspension (ml.)	0·2 M pH 7·5 borate buffer (ml.)	0·5 % trypsin (ml.)	H_2O (ml.)	Appearance of the fluid after (hr.)				
				0	0·5	3	24	72
1·0	3·0	0·45		— op.	+++ cl.	— cl.	— cl.	— cl.
1·0	3·0		0·45	— op.	— op.	— op.	++ tr.	+++ cl.

+ signs indicate the intensity of anisotropy of flow. — signs indicate no anisotropy of flow. op. = opaque, tr. = turbid, cl. = clear. The virus and trypsin (British Drug Houses) were both in water.

The action of trypsin and chymotrypsin on more highly purified preparations that have become insoluble can be spectacular. An example is given in Table 2. The virus preparation used was a colourless one that had become insoluble during dialysis. It formed an opaque suspension that slowly sedimented when allowed to stand undisturbed, and it showed no anisotropy of flow. Immediately the trypsin or chymotrypsin was added, the virus began to dissolve and anisotropy of flow began to develop, and within 30 sec. the fluids were clear and showed anisotropy of flow strongly. When incubated, anisotropy of flow decreased because of the hydrolysis of the virus, but the control incubated with borate buffer alone after 24 hr. had also begun to go into solution and to show slight anisotropy of flow. Precipitin tests can be made with the insoluble virus preparations, for they flocculate much more rapidly and at higher dilutions with virus antiserum than with saline or normal serum. Weight for weight the insoluble virus gives a smaller precipitin titre than soluble virus.

Table 2. *Effects of chymotrypsin and trypsin on an insoluble preparation of potato virus* X^S

0·5 % suspension of virus (ml.)	0·2 M pH 7·5 borate buffer (ml.)	0·25 % chymotrypsin (ml.)	0·5 % trypsin (ml.)	Appearance of the fluid after		
				30 sec.	4 hr.	24 hr.
0·4	0·4	0·2	·	+++ cl.	++ cl.	— cl.
0·4	0·4	·	0·2	+++ cl.	++ cl.	— cl.
0·4	0·6	·	·	— op.	— op.	+ tr.

Symbols as in Table 1. The virus was suspended in water: crystalline chymotrypsin and B.D.H. trypsin were dissolved in pH 7·5 borate buffer.

Treating the insoluble preparations with trypsin or chymotrypsin affects their serological reaction in various ways, corresponding with the changes in optical properties. The first effect of the enzymes, which cause the development of anisotropy of flow, leads to an increase in the precipitin titre, but with the loss of anisotropy of flow produced by hydrolysis during incubation the titre falls steadily. The development of anisotropy of flow caused by prolonged incubation with borate buffer also increases the precipitin titre, and reduces the spontaneous flocculations of the saline controls.

Table 3. *Relative susceptibility of two strains of virus X to chymotrypsin*

1·5 ml. 0·6 % solution of strain	0·2 M pH 7·5 borate buffer (ml.)	0·25 % chymotrypsin in pH 7·5 buffer (ml.)	Birefringence after incubation at 37° for (hr.)			Precipitate with CCl_3COOH		Serological titre
			0	24	48	P (mg.)	N (mg.)	
X^Y	0·65	0·35	++	−	−	0·003	0·084	< 1/4
	1·00	.	++	++	++	0·040	1·064	1/400
X^K	0·65	0·35	+++	++	++	0·014	0·420	1/200
	1·00	.	+++	+++	+++	0·028	0·952	1/400

Symbols as in Table 1.

The mechanism of re-solution is uncertain, but two alternative explanations can be offered. One is that the lateral linking of the fibres of virus *X* is brought about by impurities which are readily destroyed or replaced by trypsin and chymotrypsin. As the virus itself is broken down by trypsin, another equally likely explanation is that the splitting of a few of the entangled particles is sufficient to loosen the fibrous structure and allow the particles to separate one from the other and form a reasonably stable suspension. Electron micrograms of the insoluble preparations show the presence of material other than rod-like particles, which does perhaps support the suggestion of impurities being responsible. Also, if the phenomenon is caused by impurities, the differences between the behaviour of different preparations receives a ready explanation if we postulate different quantities of such impurities in different samples of sap. Whatever the explanation, it is clear that the interactions of virus *X* and trypsin and chymotrypsin are complex. The enzymes cause small virus particles to aggregate, whereas they disrupt large insoluble aggregates, and prolonged incubation hydrolyses the protein itself. The first two effects seem to be produced equally with the three strains, but the different strains differ considerably in their susceptibility to the proteolytic action of both trypsin and chymotrypsin. Table 3 shows the results of one experiment in which preparations of X^K and X^Y were incubated under comparable conditions with chymotrypsin. It will be seen that X^Y lost anisotropy of flow, ability to precipitate with virus antiserum and precipitability with trichloroacetic acid more rapidly than X^K. Strain X^S is intermediate. In contrast to casein, which is digested more readily by trypsin than chymotrypsin, all three strains of virus *X* are hydrolysed more rapidly by chymotrypsin than by trypsin.

Properties of nucleic acid from virus X

Sometimes the whole of the virus in a preparation becomes insoluble, but more often a part can be obtained as a colourless stable solution, spontaneously birefringent if more concentrated than 1 % and showing strong anisotropy of flow if more dilute. Preparations in this condition have been used for the preparation of nucleic acid and for testing the effects of ribonuclease. The carbohydrate content of these preparations has varied from 4 to 7 %, instead of 2·5 % found by Bawden & Pirie (1938) for their most highly purified preparations. Bawden & Pirie (1938) and Loring (1938) have described the separation of crude nucleic acid from virus *X* preparations denatured by heat or by treatment with glacial acetic acid, but did not establish its identity. We have isolated nucleic acid from virus *X* by various methods, of which the most successful was that described by Johnson & Harkins (1929) for the isolation of yeast nucleic acid. By this method 75 % of the phosphorus originally present in the virus preparation was recovered in the form of a protein-free nucleic acid. The virus solutions were incubated with 0·85 N NaOH at 0° for 2 hr., brought to pH 6 with glacial acetic acid and centrifuged free from the denatured protein. This protein contained only traces of phosphorus, but from 1·5 to 4·5 % carbohydrate, depending on the original content of the preparation. The nucleic acid was then precipitated from the clear supernatant fluid by adjusting to pH 3 with 3 N HCl and adding an equal volume of 95 % ethanol, and washed first with absolute ethanol and then with ether. The dried material contains about 7 % phosphorus, 14 % nitrogen and 33 % carbohydrate and dissolves in water at pH 5–6. The pH should be kept below 7, for at room temperature the nucleic acid depolymerizes rapidly in alkaline solutions and soon ceases to be precipitable by 0·5 N HCl. Solutions give a bluish green colour with Bial's reagent and no colour with diphenylamine reagent in acetic acid (Dische, 1930), indicating that it is a nucleic acid of the ribose, and not desoxyribose, type. Incubating a 0·1 % solution with 0·01 % ribonuclease at pH 6·5 for 3 hr. at 37° rendered the nucleic acid no longer precipitable by 0·5 N HCl. After incubation in similar conditions, and for the same length of time, without ribonuclease, half the nucleic acid was precipitable. In comparative tests with commercial preparations of nucleic acid from yeast and the nucleic acids from potato virus *X* and tobacco mosaic viruses, those from the two viruses depolymerized at the same rates with ribonuclease and with alkali alone, but the yeast nucleic acid was depolymerized more slowly. Whether this is because a different method of manufacture was used for the yeast nucleic acid or because of a real difference between the yeast and virus nucleic acids is unknown.

Reactions between ribonuclease and virus preparations

The interactions of tobacco mosaic virus and ribonuclease have been described by Loring (1942) and Kleczkowski (1946). When the two are mixed in salt-free solutions the virus is precipitated in the form of paracrystalline threads which redissolve immediately salt is added. When the two are mixed in 0·1 M borate buffer at pH 7 there is initial precipitation, but the fluids become clear after standing for 30 min. at room temperature. At concentrations at which there is no precipitation, combination between the virus and the enzyme can be demonstrated by sedimenting the virus in the ultracentrifuge. The addition of the enzyme to the virus causes an immediate drop in infectivity, but the infectivity can be restored by diluting the mixture. There is no additional effect when the mixtures of virus and enzyme are incubated. The interactions of ribonuclease and potato virus *X* in some ways resemble those of the enzyme and tobacco mosaic virus, but there are differences. Some of the differences in the behaviour with ribonuclease closely simulate the differences encountered during the purification of the two viruses.

Table 4. *Comparison of the amount of ribonuclease (RNase) combining with virus X^Y and tobacco mosaic virus (T.M.V.)*

Materials contained in 7·5 ml. H_2O at pH 6·0 (mg.)	Ribonuclease activity (as percentage of control) in	
	Supernatant fluid	Resuspended pellet
6 virus *X* + 1·5 RNase	80	20
6 virus *X*	0	0
6 T.M.V. + 1·5 RNase	23	77
6 T.M.V.	0	0
1·5 RNase	100	No pellet

The mixtures were centrifuged for 1 hr. at 40,000 r.p.m., when the supernatant fluids were decanted and the pellets were resuspended in water. The ribonuclease activities of the supernatant fluid and pellet were then estimated.

When ribonuclease and virus *X* are mixed at pH values between their isoelectric points, the fluids become opalescent, more viscous and lose their anisotropy of flow. There is no separation of obvious floccules, but low-speed centrifugation produces a sediment. There is the same phenomenon whether the solutions are salt-free or in 0·1 M borate buffer, and the addition of salt to opalescent salt-free mixtures does not increase their clarity or restore their anisotropy of flow. These viscous, opalescent mixtures resemble the insoluble fractions of virus *X* preparations that separate during the course of purification. When used in precipitin tests the mixtures give lower precipitin titres than the soluble virus, and in the presence of borate buffer they slowly become clear, recover their anisotropy of flow and original precipitin titres. The process of re-solution is greatly accelerated by the addition of chymotrypsin, though this does not cause such immediate changes as with the insoluble virus preparations used for the tests shown in Table 2. To quote one experiment: a mixture containing 0·1 % virus and 0·05 % ribonuclease in 0·05 M borate buffer at pH 7·3 became clear and regained its full anisotropy after 16 hr. at room temperature after the addition of 0·5 mg. chymotrypsin/ml., whereas the mixture in buffer alone required 36 hr. for this to occur.

As with tobacco mosaic virus, combination between virus *X* and ribonuclease can be demonstrated by mixing the two, ultracentrifuging the mixtures and assaying the supernatant fluids and resuspended pellets for their content of the enzyme. The results of one such experiment are given in Table 4, which shows that 6 mg. virus in these conditions combined with 0·3 mg. ribonuclease. In similar conditions tobacco mosaic virus combined with about four times as much ribonuclease. The comparison is of some interest, for despite the fact that weight for weight virus X combines with less of the enzyme than tobacco mosaic virus, its infectivity is more strongly inhibited by ribonuclease. Apart from quantitative differences in the amounts of ribonuclease required to cause inhibition, the inhibition of the two viruses seems to follow the same course, inhibition occurring immediately ribonuclease and virus X are mixed and infectivity being recovered by sufficient dilution. Inoculating with solutions containing 10^{-4} or 10^{-5} g. virus/ml., the concentration of ribonuclease needs to be reduced below 10^{-6} g./ml. before it ceases to cause any appreciable inhibition of infectivity. In attempts to assess any inactivation, as distinct from inhibition, by infectivity measurements, tests were made with inocula containing less than 10^{-6} g./ml. ribonuclease.

The effects of mixing ribonuclease and virus X have been reasonably constant with all the virus preparations used, but great variations have been encountered with different preparations that have been incubated with the enzyme. It is clear that ribonuclease has no effects comparable with those of the proteolytic enzymes. Incubation with concentrations of the enzyme (0·001 %) that rapidly attack the separated virus nucleic acid have no effect on the intact virus. Differences in the behaviour of preparations incubated with and without ribonuclease occur only when the enzyme concentration exceeds 0·025 %. Even at higher concentrations there was no destruction of virus when infective sap was incubated, and with no purified preparation has the virus been completely, or largely, destroyed, as it is when incubated with proteolytic enzymes. With this one exception, however, almost every possible result has been obtained with different purified preparations of the virus incubated with concentrated solutions of ribonuclease. Repeated tests with the same virus preparation have given consistent results, but using different preparations some have suffered considerable inactivation as shown by falls in infectivity and precipitin titre, whereas others have been unaffected and the rest have shown increases in both infectivity and precipitin titre. The different results are not correlated with the use of different virus strains or host plants, for all have been encountered with different preparations of strain X^K propagated in tomato plants. The preparations which have shown increases in infectivity and precipitin titre during incubation are merely reversing the fall in these two activities occasioned by the virus combining with the enzyme and separating in an insoluble form. During incubation the virus again dissolves, regains anisotropy of flow and its precipitin titre and infectivity are restored to their original. This re-solution does not seem to be brought about by any change in either the virus or enzyme, for both can be recovered with full activity. These phenomena are illustrated in Table 5, which shows that while the infectivity and precipitin titre of a virus-ribonuclease mixture were increasing during incubation, the infectivity and titre of a control solution incubated with buffer alone were falling.

Variable results have also been obtained in tests made to determine whether incubation with ribonuclease has any effect on the elementary composition of virus *X* (Table 6). After incubating virus preparations with and without ribonuclease at 37° for various periods up to 48 hr. at pH 7·5, the virus was either sedimented by ultracentrifuging or precipitated by adding an equal volume of 10 % (w/w) trichloroacetic acid and centrifuged. The pellets were then analysed for their content of phosphorus and nitrogen. In many experiments incubation with the enzyme has caused no more change in the composition of the virus than has incubation with buffer alone, but with some preparations incubation with ribonuclease has apparently caused a loss of varying amounts of phosphorus up to 30 % of that initially present. The

Table 5. *The increase of infectivity and precipitin titre when a mixture of virus X^K and ribonuclease was incubated*

Contents of mixtures			Appearance of the mixtures after (hr.)				Infectivity (numbers of lesions/leaf) after (hr.)			Serological precipitin titre after (hr.)		
0·45 % water solution of virus (ml.)	0·2 M pH 7·2 borate buffer (ml.)	0·1 % RNase in pH 7·2 buffer (ml.)	0	24	48		0	24	48	0	24	48
4·5	.	3·0	+ op.	++ tr.	++ tr.	1/50*	1	10	26	$\frac{1}{600}$	$\frac{1}{800}$	$\frac{1}{1200}$
						1/500*	2	10	12			
4·5	3·0	.	+++ cl.	+++ cl.	+++ cl.	1/50*	150		110	$\frac{1}{2500}$		$\frac{1}{1600}$
						1/500*	61		41			

RNase = ribonuclease. Symbols as in Table 1.
* Dilutions at which the mixtures were used in the infectivity tests.

Table 6. *The various effects of incubation with ribonuclease on elementary composition of different preparations of virus X^K*

	Mixtures			Precipitates with CCl_3COOH from 2·5 ml. of each mixture			
				Immediately		After 48 hr.	
Virus preparation*	0·6 % water solution of virus (ml.)	0·2 M pH 7·5 borate buffer (ml.)	0·1 % RNase in pH 7·5 buffer (ml.)	P (mg.)	N (mg.)	P (mg.)	N (mg.)
A†	3	.	3	0·032	1·11	0·021	0·97
	3	3	.	0·033	1·09	0·021	0·95
B	3	.	3	0·030	1·05	0·020	0·90
	3	3	.	0·030	1·03	0·028	0·99
C	3	.	3	0·029	0·99	0·025	0·88
	3	3	.	0·029	0·98	0·026	0·90
D	3	.	3	0·027	0·92	0·021	0·78
	3	3	.	0·027	0·91	0·022	0·79
E	3	.	3	0·030	1·10	0·022	1·00
	3	3	.	0·030	1·08	0·028	1·05

* A was isolated by fractional centrifugation alone; B, C, D and E by precipitation with acid and ammonium sulphate.

† A, from frozen sap of tomato plants; B, from frozen sap of tomato plants; C, from frozen and heated sap of tomato plants; D, from frozen sap of tobacco plants; E, from frozen and heated sap of tobacco plants.

preparations in which loss of phosphorus occurred were those in which incubation with ribonuclease also led to a drop in the precipitin titre and a loss of infectivity. Hence it does not seem that the variable results can be attributed to the presence or absence of a phosphorus-containing impurity that was attacked by the enzyme. Nor is there any evidence that such impurities occur, for the phosphorus content of crude preparations is always below that of carefully purified ones, and the apparent inactivation by ribonuclease has occurred with some of the most highly purified viruses.

No positive interpretation of these variable results can be given, but it seems most likely that ribonuclease has no enzymic activity against virus *X* and that the variations are caused by the presence of variable amounts of impurities that act as stabilizers against the injurious effects of incubation alone. Bawden & Crook (1947) have found that purified preparations of virus *X* are inactivated by incubation with phosphate buffer at pH 7 and 37° and that this inactivation is accompanied by a separation of the nucleic acid from the protein. This inactivation does not proceed to completion, but leaves a residuum of serologically active virus, and the amount of inactivation reached depends on the state of purity of the preparation. Inactivation does not occur when infective sap is incubated with phosphate and the inactivation of purified preparations can be prevented by the addition of healthy plant sap and by some protein solutions. Compared with phosphate buffer, borate caused little inactivation but this is probably the effect responsible for our variable results. When we have incubated infective sap with borate buffer, with and without ribonuclease, we have not found any loss of either infectivity or serological activity. With purified preparations, however, there has always been some loss of virus by incubation with borate buffer alone, though the amount of loss has varied from preparation to preparation. This loss is more clearly shown by the elementary analyses than by the precipitin titres or infectivity tests, as these are complicated by the virus being rendered insoluble by combining with ribonuclease and its re-solution during incubation. This phenomenon can account for the occasional rises in infectivity and precipitin titres during incubation, and probably happened in preparations containing sufficient stabilizing impurities to prevent inactivation by the borate buffer. In preparations containing less of these impurities, inactivation would proceed, and in some does proceed, sufficiently far for the beneficial effects on infectivity and precipitin titre of re-solution of the virus to be overcome, with a resulting drop in activity. Where incubation with ribonuclease has carried decomposition further than incubation with borate buffer alone, it seems likely that the enzyme has merely acted by removing degradation products of the virus and moving the state of equilibrium further towards complete inactivation.

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Synthesis and Breakdown of Glutamine by Various Micro-organisms

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SUMMARY: Glutamine is not required for staphylococcal growth, and was found to be synthesized by staphylococci from NH_3 and glutamic acid in the presence of glucose. The amount of glutamine found was, however, much less than that of the NH_3 reacting. Similar relationships were found in *Proteus morganii*, *Pr. vulgaris*, *Saccharomyces cerevisiae* and *S. ellipsoideus*. *Proteus morganii* was, however, outstanding in possessing a powerful glutaminase which could easily be separated from the cells; in this it differed markedly from *Pr. vulgaris*. Glutaminase activity was also demonstrated in yeasts, where its occurrence has been previously a matter of dispute.

Glutamine is essential for growth of certain β-haemolytic streptococci; it accelerates their main energy-yielding reaction, glycolysis, and it is hydrolysed to glutamic acid and NH_3 during this process (McIlwain, 1946*a*, *b*). Certain of these associations may be explained by the need of energy for the assimilation of glutamic acid and glutamine (Gale, 1947). This requirement (Taylor, 1947), like that for glutamine in growth (McIlwain, Fildes, Gladstone & Knight, 1939), is different in different microbial species. We have now studied the association between glucose, glutamine, glutamic acid and ammonia in several micro-organisms.

METHODS

Organisms. Staphylococcal strains comprised '35', from a case of osteomyelitis and 'E' from one of impetigo, and were grown under the conditions described for streptococci by McIlwain (1946*a*, *b*). Strains of *Proteus morganii* included No. 2818 of the National Collection of Type Cultures, and two freshly isolated from faeces; they were grown as described by McIlwain & Hughes (1945), but with 10^{-6} M pantothenate. *Pr. vulgaris* was grown as *Pr. morganii*; one strain (C) was from an infected wound, and the other had been maintained on laboratory media for many years. *Saccharomyces cerevisiae* and *S. ellipsoideus* were respectively Nos. 4614 and 7040 of the National Collection, No. 7040 being used shortly after its receipt from a distillery. They were grown in a mixture of inorganic and ammonium salts with 2 % glucose and 1–5 % of a bacteriological infusion broth. Bacteria were maintained on broth agar slopes and grown at 37°; yeasts, on malt-agar slopes and at 25° except where otherwise described.

Experimental procedure. The metabolic experiments were made and NH_3 and glutamine were determined by methods stated previously (McIlwain, 1946*a*, *b*; Roper & McIlwain, 1948). Yeasts were extracted mechanically and autolytically by the methods applied to bacteria (McIlwain, Roper & Hughes, 1948). The saline used was that of Krebs & Eggleston (1940).

RESULTS

Staphylococci

Growth. Glutamine (5×10^{-6} M to 5×10^{-4} M) was without effect on growth of the two strains under conditions in which the substance is necessary for streptococcal growth. Slight stimulation has been found in other strains (cf. Fildes & Gladstone, 1939), but was not sufficiently well defined to afford a subject for investigation. The sulphoxide and sulphone derived from methionine were without effect on the growth of strain 35, when present at 0·01 M in an amino-acid medium (Fildes & Richardson, 1937). (Inhibition is caused by the methionine derivatives in other bacteria and is related to their metabolism of glutamic acid.)

Table 1. *Reaction of staphylococci with glutamine and ammonium glutamate*

Strain of organism: dry wt. (mg.); duration of experiment (min.)	Substrates (μmol.)	Change (μmol.) in		
		Glutamine	NH_3	CO_2
'35'; 4·7; 80.	Glutamine, 15	−2·0	+2·3	+ 1·8
	Glutamine, 15 and glucose, 200	−0·6	<0·1	+82
	Ammonium glutamate, 31	+0·1	<0·1	+ 1·0
	Ammonium glutamate, 31 and glucose, 200	0	−4·2	+82
'35'; 2·5; 120	Glutamine, 12·6	−2·2	+2·2	+ 1·2
	Glutamine, 12·6 and glucose, 200	<0·1	<0·1	+54
	Glucose, 200	<0·1	<0·1	+54
'35'; 2·1; 120	Ammonium glutamate, 95·5 and glucose, 200	<0·1	−6·5	+53
	Ammonium glutamate, 24·0 and glucose, 200	<0·1	−4·8	+54
'E'; 6; 80	Glutamine, 14·4	−0·9	+0·9	+ 0·8
	Glutamine, 14·4 and glucose, 200	<0·1	<0·1	+35
	Ammonium chloride, 15·1 and glucose, 200	<0·1	−1·0	+36

Experiments were performed anaerobically at pH 7·6 and 37° with the substrates and amount of organism indicated, in 3 ml. of bicarbonate saline. The changes recorded are in the complete metabolic mixtures without separation of organisms. Organisms were grown for 18–22 hr., washed twice with 0·9 % NaCl, and suspended in that solution for use.

Anaerobic reactions with glucose. The reaction was examined only in order to find whether glutamine affected it, as is the case with streptococci. Acid was formed from glucose and determined (*a*) by evolution of CO_2 from bicarbonate which occurred at rates comparable to those observed in streptococci; and (*b*) as lactic acid, according to Friedemann & Graeser (1933). Table 1 includes experiments in which the rates were of 10–15 μmol./mg. dry wt. of cells/hr. Glutamine had little or no effect on the rates, the maximum increase of some 8 % being no more than that given by ammonium glutamate. The organisms were examined after 5, 18 and 30 hr. growth.

Action on glutamine and ammonium salts. Glutamine was decomposed with formation of NH_3 in the absence of glucose (Table 1), a phenomenon not found in the exacting streptococci. The rate of decomposition was, however, not high; mean values for $Q_{\text{glut.}}$ calculated from the data of Table 1, assuming the rate to be steady during the experiments, were $-0{\cdot}12$ to $-0{\cdot}44$ μmol./mg. dry wt./hr., or about 1/5 to 1/10 of those observed in streptococci in the presence of glucose. The NH_3 formed was about equivalent to the glutamine decomposed; the organisms may thus be said to exhibit a small 'glutaminase' activity. (The term 'glutaminase' has been retained in describing such formation of glutamic acid and NH_3 from glutamine, though it may later be necessary to use a more specific name such as 'glutamine desamidase'. 'Glutaminase' has been avoided in describing the coupled breakdown of glutamine to the same products, which occurs, for example, during streptococcal glycolysis.) During this reaction the total CO_2 evolved from the bicarbonate containing media was small, being less than the change in glutamine. No reaction corresponding, for example, to glycolysis from stored carbohydrate, was therefore occurring.

In marked contrast to the findings with streptococci, glucose did not increase the quantity of ammonium glutamate formed from glutamine, but decreased it. The ratio of change in glutamine to change in CO_2 then became $<1:100$ and often $<1:500$ (Table 1), whereas the corresponding ratios with exacting streptococci were 1 : 5 to 1 : 20. Such decomposition of glutamine as did occur with staphylococci in the presence of glucose did not lead to accumulation of NH_3.

Ammonia added as NH_4Cl or as ammonium glutamate also disappeared in the presence of glucose; the rate of reaction was then $-0{\cdot}7$ to $-1{\cdot}6$ μmol. NH_3/mg. dry wt./hr. The glutamine formed was too small to be detected by the method of Vickery, Pucher, Clark, Chibnall & Westall (1935), but could be determined microbiologically. By such methods (cf. Roper & McIlwain, 1948) the strain 'E' in phosphate buffer of pH 7·6, with glucose (0·017 M) and ammonium L-glutamate (0·0053 M) for short periods (cf. McIlwain *et al.* 1948) synthesized and stored glutamine in the cells, usually at rates of 5–15 mμmol./mg. dry wt./hr. In one case a rate of 50 mμmol./mg./hr. was observed. These indicate minimum values only for the rate of glutamine synthesis.

Proteus morganii

Growth. Glutamine (10^{-5}–10^{-3} M) did not affect the rate of growth of *Pr. morganii* under the conditions in which this substance was essential to streptococci, nor under the conditions employed in growing the organisms for the present metabolic experiments.

Action on glucose. The two strains rapidly produced acid from glucose anaerobically and their Q_{CO_2} in bicarbonate-containing media was equal to, or greater than, that of the streptococci. Added glutamine did not affect their Q_{CO_2}.

Action on glutamine and ammonium salts. Pr. morganii caused a rapid breakdown of glutamine both with and without glucose. Without glucose, NH_3 was formed in approximately equimolar quantities (Table 2), but with glucose, much less NH_3 was found. This appeared to be due to a secondary reaction.

The disappearance of NH_3 from glutamine was not confined to NH_3 from this source; NH_3 production from asparagine was also less in the presence of glucose than in its absence. The strain 2818 produced NH_3 anaerobically and in the absence of glutamine, at pH 7·6, at the rate of 2·9 μmol./mg./hr. from asparagine, and at 2·8 μmol./mg./hr. from aspartic acid; the reactions, if any, with pyrrolidone-α-carboxylic acid or with arginine gave $<0{\cdot}2$ μmol. NH_3/mg./hr. Ammonia added as NH_4Cl to *Pr. morganii*, in the presence of glucose, also disappeared at about the same rate as did that from glutamine (Table 2), though in the absence of glucose little change occurred in NH_3. The disappearance of added NH_3 during reaction with glucose was frequently less in the presence of glutamic acid, but ammonium glutamate yielded no glutamine either in the presence or absence of glucose. It is understandable that little or no glutamine should accumulate as such (though the organisms presumably synthesize and assimilate it in growth), because its breakdown is rapid.

Table 2. *Reaction of* Pr. morganii *with glutamine and ammonium salts*

Experimental arrangement as described in Table 1

Strain of organism; dry wt. (mg.); duration of experiment (min.)	**Substrates (μmol.)**	**Change (μmol.) in**		
		Glutamine	**NH_3**	**CO_2**
'2818'; 3·1; 35	**Glutamine, 14·1**	**−13·8**	**+14·0**	**+ 2**
	Glucose, 200	**0**	**0**	**+59·5**
	Glucose, 200 and glutamine, 14·1	**−12·4**	**+ 8·1**	**+58·5**
	Glucose, 200 and glutamine, 5·6	**− 5·3**	**0**	**+61**
'2818'; 5·1; 35	**Glucose, 200**	**0**	**0**	**+55**
	Glucose, 200 and ammonium glutamate, 15	**0**	**− 6·7**	**+54·5**
	Glucose, 200 and ammonium chloride, 15	**0**	**− 7·0**	**+55·5**
	Ammonium glutamate, 15	**0**	**+ 0·2**	**0**
	Ammonium chloride, 15	**0**	**+ 0·2**	**0**
Lab. strain 2; 3·8; 45	**Glutamine, 15·8**	**−14·8**	**+15·0**	**+ 0·5**
	Glutamine, 15·8 and glucose	**−14·5**	**+ 8·4**	**+53**
	Ammonium glutamate, 14·8 and glucose	**0**	**− 3·2**	**+51**
	Ammonium chloride, 15·8 and glucose	**0**	**− 7·1**	**+54**
	Ammonium chloride, 15·8	**0**	**− 0·2**	**+ 0·5**
	Glucose, 200	**0**	**0**	**+54**

Pr. morganii thus differed from the staphylococci in its much more rapid glutaminase action, and in the fact that such action was unaffected by added glucose. The disappearance of NH_3 in the presence of glucose but without corresponding glutamine synthesis (a feature common to both these organisms and many others) was carried out more rapidly by *Pr. morganii*.

Extraction of a glutaminase from Pr. morganii. The cells were easily broken by ground glass and in the preparation used for the experiments of Table 3, 99 % of the cells had been broken. The extract had about one-third of the activity manifested by the intact cells at pH 7·6; the debris had one-quarter the activity. The remainder was not necessarily lost as the pH of the test system was not that which was later found to be optimal for glutamine breakdown. The extract was much more active at pH 5.

Autolysis also yielded an active solution, containing some 60 % of the activity of the cells from which it was prepared. Like the intact cells this yielded 1 mol. NH_3/mol. glutamine decomposed (Table 3).

Table 3. *Ammonia formation from glutamine by extracts of* Pr. morganii

Preparation; equivalent dry wt. (mg.) of bacteria used	Buffering	NH_3 produced (μmol.) in 1 hr.	Glutamine lost (μmol.) in 1 hr.
Fresh cells; 3	Bicarbonate, pH 7·6	18·6	—
First extract of ground cells; 3·2	Bicarbonate, pH 7·6	4·0	—
Second extract of ground cells; 3·2	Bicarbonate, pH 7·6	2·6	—
Ground cell residue; 7·0	Bicarbonate, pH 7·6	3·6	—
First extract of ground cells; 3·2	Acetate, pH 4·5	22·0	—
Fresh cells; 2	Phosphate-citrate, pH 4·5	55·0	—
Autolysate; 2	Phosphate-citrate, pH 4·5	40·8	—
Residue from autolysis; 2·5	Phosphate-citrate, pH 4·5	25·3	—
Autolysate (different preparation); 2	Phosphate-citrate, pH 4·5	35·6	35·7
Autolysate (different preparation); 2	Phosphate-citrate, pH 4·5	35·6*	34·9*
Autolysate (different preparation); 2	Phosphate-citrate, pH 2·5	25·6	26·6

* Aerobic reaction.

Extracts were prepared from strain 2818 after 18 hr. growth. Reactions were performed anaerobically unless otherwise specified, and with initially 60 μmol. glutamine per reaction mixture of 3 ml. Controls at the experimental pH values showed no spontaneous breakdown of glutamine.

Proteus vulgaris

Growth. In the casein hydrolysate medium employed in growing *Pr. vulgaris* for the present experiments, addition of glutamine (10^{-5}–5×10^{-4} M) did not alter the mass of bacteria produced. The medium contained glutamic acid which was observed by Fildes & Gladstone (1939) to stimulate growth of a strain to almost the same extent as did glutamine when the substances were added to a medium of inorganic salts and lactate.

Action on glucose. The two strains produced acid from glucose. Carbon dioxide was evolved from bicarbonate-containing solutions at rates of 6–15 μmol./mg. dry wt./hr. and these rates were increased by about 0·008 M ammonium salts or by glutamine to small and equal extents. Thus the strain B (Table 4), when evolving CO_2 at the rate of 15·8 μmol./mg./hr., was stimulated 16 % by glutamine and 13 % by ammonium glutamate; in strain C, the rate of 16·1 μmol./mg./hr. was increased 14 % by both compounds.

Action on glutamine and ammonium salts. Table 4 shows that, in marked distinction from the behaviour of *Pr. morganii*, *Pr. vulgaris* had little action on glutamine alone. Ammonia disappeared only slowly, without formation of glutamine, from solutions of ammonium glutamate added to suspensions of *Pr. vulgaris*, but this reaction and the decomposition of glutamine became considerable in the presence of glucose. Here again, metabolism of glucose was associated with a reaction leading to loss of the labile amide—NH_3 of glutamine, at a rate of 1–2 μmol./mg. dry wt./hr., but without accumulation of NH_3. Acetyl phosphate did not induce breakdown of glutamine but led to loss of NH_3; the CO_2 produced (Table 4) during the experiments corresponded approximately to the compound's spontaneous hydrolysis (Lipmann & Tuttle, 1944).

The two strains were typical of *Pr. vulgaris* in exhibiting powerful urease activity.

Table 4. *Reaction of* Pr. vulgaris *with glutamine and ammonium salts*

Experimental arrangements as described in Table 1, except that pH was varied as indicated below.

Strain of organism; dry wt. (mg.)	Duration of experiment (min.)	pH	Substrates (μmol.)	Change (μmol.) in		
				Glutamine	NH_3	CO_2
B; 8·2	60	4·5*	Glutamine, 18·7	0	0	+ 0·7
	60	7·6	Glutamine, 18·7	0	+ 0·8	+ 1·1
	60	7·6	Glutamine, 18·7 and glucose. 200	−5·9	0	+42·2
C; 7·2	60	4·5*	Glutamine, 18·7	−1·0	0	+ 0·6
	60	7·6	Glutamine, 18·7	+0·2	+ 1·2	+ 1·0
	60	7·6	Glutamine, 18·7 and glucose, 200	−5·1	0	+45·0
B; 6·35	100	7·6	Ammonium glutamate, 45·5	0	− 3·1	+ 0·5
	30	7·6	Ammonium glutamate, 45·5 and glucose, 200	0	− 6·5	+55·3
	30	7·6	Glutamine 21, and glucose, 200	−5·4	+ 0·2	+57·8
	100	7·6	Glutamine, 21	0	+ 0·3	+ 0·6
	30	7·6	Urea, 25	0	+33·6	—
C; 6·95	100	7·6	Ammonium glutamate, 27	0	− 3·3	+ 0·3
	35	7·6	Ammonium glutamate, 27 and glucose, 200	0	− 6·3	+50·1
	100	7·6	Ammonium glutamate, 27 and acetyl phosphate, 50	0	− 4·3	+10·9
	100	7·6	Glutamine, 15·7	−0·8	+ 0·5	+ 0·7
	100	7·6	Glutamine, 15·7, and acetyl phosphate, 50	−0·8	+ 0·5	+12·2
	30	7·6	Glutamine, 15·7 and glucose, 200	−7·8	+ 0·3	+58

* Acetate buffer used instead of bicarbonate.

Yeasts

The formation of NH_3 from glutamine by yeast extracts was reported by Geddes & Hunter (1928) and Grassman & Mayr (1933), without experimental details and in studies incidental to those of yeast asparaginase. Archibald (1945) found no glutaminase activity and stated that this was also the finding of Schwab (1936) and Nielsen (1941). The observations given below indicate well-marked glutaminase action, but show that the presence or absence of glucose greatly affects the reaction of yeasts to glutamine. Also, glutamine can be broken down without accumulation of NH_3.

Growth. The effect of glutamine in concentrations between 10^{-5} M and 3×10^{-4} M on growth of the strains of *Saccharomyces cerevisiae* and *S. ellipsoideus* at 25 and 35° was examined in media consisting of ammonium salts, inorganic salts and glucose, and in the mixture of amino-acids used by Gladstone (1939). In the different experiments, with varying inocula, visible growth first appeared at 1–4 days, but in no case was a significant acceleration by glutamine observed.

Fermentation. The production of CO_2 from glucose by baker's yeast was observed by Smythe (1939) to be accelerated by glutamine, an effect which he concluded was mediated through NH_3 formed from the glutamine. The present observations are in agreement with this. Stimulation of CO_2 production at pH 7·5 by glutamine was smaller (4–8 %) than by ammonium glutamate (12–20 %). The yeasts were grown for periods between 30 and 72 hr. and were washed in saline, but were not prepared by the methods described by Smythe to obtain maximal response to NH_3.

Action on glutamine and ammonium salts. Reaction mixtures with and without glucose were examined for anaerobic production or disappearance of ammonia and glutamine (Table 5). The values show that rarely, if ever, did a simple conversion of glutamine to glutamic acid and NH_3, or the reverse reaction, take place. Decomposition of glutamine was slow, except in the presence of glucose. With glucose it reached values of 1 μmol./mg./hr., and thus approximated to the rate of glutamine decomposition by streptococci. Unlike the reaction with streptococci, however, little NH_3 accumulated as a result of the loss of the labile amide group of glutamine in the presence of yeasts. Added NH_3 also disappeared either at a slightly greater (*S. ellipsoideus*) or much greater rate (*S. cerevisiae*) than that of the reaction with glutamine. Any NH_3 formed from glutamine would therefore be expected to undergo further reaction and not to accumulate. The two yeasts differed, not in their rates of reaction with ammonium salts, but in their rates of glutamine decomposition, which was lower in *S. cerevisiae*. A small accumulation of synthesized glutamine was observed in some cases (Table 5).

Yeast extracts and glutamine. An extract prepared by rubbing moist cells of *S. ellipsoideus* with powdered glass contained little free glutamine and NH_3, and on incubation anaerobically with added glutamine at pH 4·5 or 7, it caused a slow breakdown of glutamine ($-0{\cdot}2$ to $-0{\cdot}3$ μmol./quantity of extract prepared from the equivalent of 1 mg. dry wt. of yeast/hr.). The rate was unaffected by glucose. The molar quantity of NH_3 produced was less than that of the glutamine lost.

On autolysis in the presence of fluoride, extracts with smaller glutaminase activity were obtained. These decomposed 0·1–0·2 μmol. glutamine/mg.-equivalent/hr. with the formation of about an equimolar quantity of NH_3. Such activity may account for the reports of Geddes & Hunter (1928) and Grassmann & Mayr (1933); it represents, however, only about one-tenth of the activity exhibited towards glutamine in the presence of glucose.

Table 5. *Reactions of yeasts with glutamine and ammonium glutamate*

Experimental arrangements as described in Table 1, except that pH was adjusted as indicated below, and the organisms were from cultures harvested at 48–52 hr. after inoculation.

Organism; dry wt. (mg.); duration of experiment (min.)	Buffering	Substrates (μmol.)	Change (μmol.) in		
			Glutamine	NH_3	CO_2
S. ellipsoideus; 8·8; 45	Bicarbonate, pH 7·6	Glutamine, 15	0	+0·7	+ 1
	Bicarbonate, pH 7·6	Glutamine, 15 and glucose, 200	−5·8	0	+120
	Bicarbonate, pH 7·6	Ammonium glutamate, 15	+0·5	−0·3	+ 2
	Bicarbonate, pH 7·6	Ammonium glutamate, 15 and glucose, 200	0	−7·9	+135
S. ellipsoideus; 7·5; 45	Phosphate-citrate, pH 6·5	Glutamine, 20	0	+0·3	+ 1
	Phosphate-citrate, pH 6·5	Glutamine, 20 and glucose, 200	−5·9	0	+ 64
	Phosphate-citrate, pH 4·5	Glutamine, 20	−2·0	0	+ 1
	Phosphate-citrate, pH 4·5	Glutamine, 20 and glucose, 200	−3·1	0	+ 88
S. ellipsoideus; 8·7; 35	Phosphate-citrate, pH 4·5	Ammonium glutamate, 36	0	+0·5	—
	Phosphate-citrate, pH 4·5	Ammonium glutamate, 36 and glucose, 200	0	−6·9	+ 74
	Phosphate, pH 7	Ammonium glutamate, 36	0	−0·3	—
	Phosphate, pH 7	Ammonium glutamate, 36 and glucose, 200	0	−6·5	+ 74
	Phosphate, pH 7	Ammonium glutamate, 36 and glucose, 200 and fluoride, 250	0	+0·5	+ 0·1
	Phosphate, pH 7	Ammonium glutamate, 36 and glucose, 200 and fluoride, 50	0	−4·8	+ 44
S. cerevisiae; 6·2; 65	Bicarbonate, pH 7·6	Glutamine, 10·6	+0·2	+0·1	+ 1
	Bicarbonate, pH 7·6	Glutamine, 10·6 and glucose, 200	−1·8	0	+ 63
	Bicarbonate, pH 7·6	*dl*-Ammonium glutamate, 18·5	+0·2	0	+ 0·6
	Bicarbonate, pH 7·6	*dl*-Ammonium glutamate, 18·5 and glucose, 200	0	−4·8	+ 65
S. cerevisiae; 2·9; 60	Phosphate-citrate, pH 6·5	Glutamine, 25	−2·4	+1·0	+ 0·3
	Phosphate-citrate, pH 5·5	Glutamine, 25	−0·4	+0·2	+ 0·2
	Phosphate-citrate, pH 4·5	Glutamine, 25	+0·1	0	+ 0·1
	Phosphate-citrate, pH 4·5	Glutamine, 25 and glucose, 200	−3·4	0	+ 26·3

DISCUSSION

The changes which have been observed in glutamine and in ammonium glutamate, in the presence and absence of glucose, are summarized in Table 6. All the organisms are capable of reactions involving either the formation or the removal of NH_3 (or both reactions) at rates of at least 1–2 μmol./mg. dry wt./hr. In straightforward hydrolysis of glutamine to glutamic acid and NH_3, *Proteus morganii* is outstanding. Its reaction, at the rate of 6–8 μmol./mg./hr., results in decomposition in an hour of about its own weight of glutamine. Comparable reactions were not found in the other organisms, and *Pr. morganii* is seen to differ even from *Pr. vulgaris*. Rapid glutaminase action has also been observed in strains of *Clostridium welchii* (Krebs, 1948). It was easy to separate the glutaminase of *Pr. morganii* in cell-free form.

Table 6. *Summary of reactions of micro-organisms with glutamine and ammonium glutamate*

		Quotients* (μmol./mg. dry wt./hr.) with			
		Glutamine		Ammonium glutamate	
Organisms		Alone	With glucose	Alone	With glucose
β-Haemolytic streptococci	Q_{NH_3}	0 to +0·1	+1 to +3	<0·03	0 to −0·08
	$Q_{glut.}$	0 to −0·1	−1 to −3	<0·03	<0·03
Yeasts	Q_{NH_3}	0 to +0·03	0·02	0 to −0·03	−0·7 to −1·2
	$Q_{glut.}$	<0·02	−0·3 to −1	+0·02 to +0·05	<0·02
Staphylococci	Q_{NH_3}	+0·1 to +0·4	<0·02	0 to −0·02	−1 to −1·5
	$Q_{glut.}$	−0·1 to −0·4	0 to −0·1	0 to +0·02	<0·02
Pr. morganii	Q_{NH_3}	+6 to +8	+4	<0·02	−1·5 to −2·5
	$Q_{glut.}$	−6 to −8	−6 to −8	<0·02	<0·02
Pr. vulgaris	Q_{NH_3}	0 to 0·15	0 to +0·1	−0·3	−1·6 to −2·1
	$Q_{glut.}$	0 to −0·1	−0·7 to −2·2	<0·03	<0·03

* Changes have been expressed as metabolic quotients for convenience in summarizing, but only in the cases of the haemolytic streptococci, staphylococci and *Pr. morganii* have some kinetic experiments been carried out; the remaining values are calculated from overall exchanges in experiments of the type of those of Tables 1–5. The experimental arrangements were similar throughout; reactions were carried out anaerobically at pH 7·6.

The glutaminase of *Pr. morganii* is characteristically distinct from the system in the exacting haemolytic streptococci, which decomposes glutamine to glutamic acid and NH_3 during glycolysis (McIlwain, 1946*a*, *b*). In this system extracts capable of hydrolysis of glutamine were not obtained (McIlwain *et al.* 1948), and it is possible that the true substrate of the streptococcal enzyme might not be glutamine, or that the reaction might be a phosphorolysis rather than hydrolysis. Precise evidence is therefore required before the term 'glutaminase' can be applied to such a system, and before the relation between glutamine breakdown and glycolysis can be regarded as due only to permeability phenomena (cf. Gale, 1947). Breakdown of glutamine, associated with glycolysis, has now been demonstrated in yeasts and in *Pr. vulgaris*, so that its occurrence is independent of assimilatory mechanisms peculiar to Gram-positive organisms.

One further feature of those haemolytic streptococci that are exacting towards glutamine in growth is their relative inertness towards ammonia, either added as such or produced during metabolism. Thus the ammonia formed from glutamine by these organisms, in the presence of glucose, was almost equivalent to the glutamine decomposed (McIlwain, 1946*a*), whereas in the case of other organisms it was much less (Table 6). This was true even with *Pr. morganii* which produced such NH_3 very rapidly. The equivalence, in streptococci, may be a reflexion of the relative lack in these organisms of synthetic reactions using NH_3. The streptococci are nutritionally the most exacting of the organisms of Table 6, and require many preformed amino-acids, for their growth.

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Recent Experiences in the Rapid Identification of *Bacterium coli* Type I

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SUMMARY: When using MacConkey broth medium incubated at 44° as a confirmatory test for *Bacterium coli* Type I, positive reactions were produced by anaerobic lactose-fermenting bacteria such as *Clostridium welchii*. The use of brilliant green bile broth instead of MacConkey broth was completely successful in suppressing the growth of these organisms at 44°, but did not influence the growth of *Bact. coli* Type I. Two other coliform organisms, namely, Irregular Type II and Irregular Type VI were found to ferment lactose at 44°. A rapid test for their differentiation from *Bact. coli* Type I has been based upon the ability of that organism to produce indole at 44°, whereas the two irregular types are indole negative. By subculture of presumptive positive tubes in brilliant green bile broth and peptone water together with incubation at 44°, an estimation of the *Bact. coli* content of a water sample may be obtained within 48 hr. of primary inoculation.

It is now over 40 years since Eijkman showed that coliform bacteria from the intestines of warm-blooded animals would grow and produce gas in glucose broth at a temperature of 46°, whereas coliforms from the intestines of cold-blooded animals would not. Modifications of the Eijkman test with regard to medium and temperature have been introduced from time to time and Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935) considerably modified the test and recommended its use for the routine detection of *Bacterium coli* (*Escherichia coli*) in samples of milk. The test, as then modified, consisted of fermentation of MacConkey broth at 44° in an accurate, thermostatically controlled water-bath. It has the advantage over the older plating method of considerable saving in time, labour and cost. The method was advocated for the detection of *Bact. coli* Type I in water analysis by Mackenzie & Hilton-Sergeant (1938), and a further series of comparative tests were reported by Mackenzie (1938). The test was officially recommended for routine work in the Ministry of Health Report, No. 71 (1939). A full bibliography was published by Batty-Smith (1942) so that further reference to the literature is unnecessary.

Two procedures were suggested by Wilson *et al.*, either the direct inoculation of the milk sample into tubes of media and incubation at 44°, or the subculture of primary positive tubes from the first 24 hr. at 37° into MacConkey medium and incubation of these at 44°. It was found (Mackenzie, 1938) that for river-water samples, where *Bact. coli* was abundant, primary incubation at 44° was as efficient as the subculture procedure; but when stored, filtered and chlorinated water samples were tested, the organisms, possibly because of attenuation, were more frequently recovered by the subculture procedure than by direct inoculation at 44°. Since, in addition, very large incubator baths would have been needed to accommodate the groups of tubes to be set up for each test, the subculture procedure for the rapid identification of *Bact. coli* was chosen for our laboratories and first used as a routine test in September 1939. It is now being employed successfully at many other laboratories in Great Britain and elsewhere.

This paper contains an account of experiences with the 44° test, and of certain modifications that have been introduced from time to time which have considerably enhanced the value of the test as a means for the rapid identification of *Bact. coli* Type I.

Water-bath at 44°

An essential feature of the test is that the medium should be maintained at the prescribed temperature with only very slight deviation. If this cannot be done it is inadvisable to use the test. In our laboratories an almost constant temperature has been achieved and a detailed description of the method used may therefore be of value.

A copper bath, provided with a closely fitting lid, is set up in a thermostatically controlled cupboard, the temperature of which is then regulated empirically to maintain the water in the bath at 44°. The temperature is recorded by a thermometer immersed in the bath which gives a continuous record on a chart situated outside the incubator rooms. The record indicates whether any electrical breakdown has occurred during the period of incubation. In addition, an accurate thermometer calibrated to 1/50th of a degree Centigrade is kept in the bath and is observed once daily as a control. The cupboard and bath are opened only twice daily, with precautions to prevent cold air entering the apparatus, the cupboard being approached through a heated and thermostatically controlled entrance chamber. After inoculation, the tubes are rapidly heated to 44° in a separate water-bath before being placed in the special incubator. Fifty ml. water, previously heated to 44°, are added to the bath periodically to replace that lost by evaporation. When all these precautions are observed the temperature deviation never exceeds 0·1°.

Incubation in brilliant green bile broth at 44°

In all the work recently described single strength MacConkey broth medium has been employed for the test. When using this medium we noticed that subcultures from some presumptive positive tubes gave vigorous gas production at 44° but only slight acidity, the litmus indicator changing to purple rather than to red or orange, and that when these presumptive positive tubes were plated on MacConkey agar no growth occurred. This reaction—'purple' acidity with good gas production—occurred most frequently in chlorinated waters and proved to be caused by anaerobic lactose fermenting organisms. *Clostridium welchii* (*Cl. perfringens*) and other bacteria of this group could be isolated from 5 to 10% of all positive tubes from samples of London waters, and it was the cultures from these tubes which gave the 'purple' reaction.

In an endeavour to overcome this disadvantage, tests were made of several different media and two, brilliant green bile broth and standard MacConkey broth, were selected for detailed comparison. The brilliant green bile broth was made up as follows (American Public Health Association, 1936): Bacto ox gall, 30 g.; Bacto peptone, 15 g.; lactose, 15 g.; Bacto brilliant green (1 % w/v), 2 ml.; and distilled water to 1500 ml., the pH being adjusted to 7·2–7·4.

Each day, a number of presumptive positive tubes were selected from the routine work, particular care being taken to include tubes likely to produce the 'purple' reaction in MacConkey broth at 44°, and subcultures made into single-strength MacConkey broth and into single-strength brilliant green bile broth. Both series of tubes were incubated in the 44° water-bath for 24 hr. Positive results in MacConkey broth were indicated by the production of acid and gas; positive results in brilliant green bile broth were indicated by gas production but there was, of course, no change in colour as occurs in MacConkey broth. Occasionally gas production was slight in brilliant green bile broth, and could be demonstrated only by sharply tapping the tube with a ruler or pencil. Cultures exhibiting any degree of gas production in this medium, however slight, almost invariably proved to contain *Bact. coli* Type I. In all, 1419 tubes positive in the presumptive coliform test were tested in this way. Table 1 shows the number of positive reactions obtained with each medium, and the number which was subsequently proved to have been due to *Bact. coli* Type I (acid and gas in lactose, indole positive, no growth in citrate, methyl red test positive, Voges-Proskauer test negative and 44° test positive).

Table 1. *Comparison of results of subculture of* 1419 *primary positive cultures into brilliant green bile broth and MacConkey broth at* 44°

	No. of subcultures reacting positively at 44°	No. of positive subcultures yielding *Bact. coli* Type I
MacConkey broth	431	314 (72·8 %)
Brilliant green bile broth	319	313 (98·1 %)

Brilliant green bile broth was markedly superior to MacConkey broth for confirmation of *Bact. coli* Type I at 44° and was adopted as the routine medium on 1 June 1941. Thousands of positive tubes have since been plated and the organisms responsible for the positive reactions identified. In no case has a positive reaction been found to be due to an anaerobic lactose-fermenting bacterium.

Irregular coliform types growing at 44°

Beside *Bact. coli* Type I, two other coliform types can grow at 44°, namely Irregular Type II and Irregular Type VI. Their primary habitat has not, however, been established as faecal.

Irregular Type II. Its reactions are acid and gas in lactose, indole negative, no growth in citrate, methyl red test positive, Voges-Proskauer test negative, 44° test positive.

It is an indole negative variety of *Bact. coli* Type I and is classified by Wilson *et al.* (1935) as Irregular Type II. Its frequency in human faeces is low. For example, from a series of 780 specimens from apparently healthy individuals, examined by the authors during routine examinations for typhoid carriers, Irregular Type II was found in only 20 (2·6 %). Comparative figures for

Intermediates (*Escherichia freundii*) and *Bacterium aerogenes* (*Aerobacter aerogenes*) were 30 (3·8 %) and 8 (1·0 %) respectively.

Unfortunately, the majority of the strains from human excrement were isolated during the war when circumstances precluded a detailed study of all of them. A special study of sixty-four strains of Irregular Type II has, however, been made, fifty-nine isolated from water and five from faeces. The cultures were maintained for 6 years by plating alternately on nutrient gelatine and agar media at intervals of 2–3 months. From time to time the reactions of the strains were re-examined, with the following results (Table 2): (1) all reactions remained constant for the whole 6 years, except indole production; (2) a high proportion of the strains isolated from water became indole positive; (3) none of the faecal strains became indole positive.

Table 2. *Acquisition of power to produce indole by* Bacterium coli *Irregular Type II*

No.	Source	No. becoming indole positive
59	Water	21 (35·6 %)
5	Faeces	0

Length of time before change took place:

No.	Time
13	Within 12 months
6	1–2 years
2	3–5 years

Each of these mutating strains was carefully investigated, and in all cases it was found that an indole negative strain produced a mixture of indole positive and indole negative colonies on subculture, the former breeding true indole positives on further subculture, whilst the latter again gave off a mixture of indole positive and negative varieties. Table 3 illustrates a typical experience; the strain was plated out and thirty colonies were subcultured, each into a large tube of peptone water which was tested for indole at intervals. It will be seen that one single-colony strain remained obstinately indole negative, but after several subcultures over a year, a peptone water subculture from the stock agar slope gave a positive indole reaction, indicating that mutation was still taking place.

Table 3. *Indole production at 37° in peptone water subcultures of thirty single colonies of a strain of* Bacterium coli *Irregular Type II*

Days	2	4	6	8	10	14	21	28
Number of positive reactions in thirty subcultures	1/30	2/30	10/30	17/30	19/30	21/30	27/30	29/30

When this coliform type mutates to an indole positive variety it is indistinguishable from *Bact. coli* Type I, and there seem, therefore, good grounds for considering it as a faecal type. On the other hand, no mutation was observed in any of the five strains actually isolated from faeces. The number examined is admittedly small, and more work is required on these intestinal strains. Another view is that the mutating specimens isolated from water are attenuated *Bact. coli* Type I strains which revert to their true reactions under better environmental conditions. If such is the case, then again Irregular Type II should be considered as a true faecal type. A somewhat paradoxical situation thus arises. Irregular Type II, when isolated from water, may mutate when subcultured for a sufficiently long period, and become indistinguishable from *Bact. coli* Type I. An organism producing the same original reactions to all commonly used tests, when isolated from the animal intestine, has retained the characteristic which distinguishes it from *Bact. coli* Type I. From this it might be argued that Irregular Type II isolated from water is a typical faecal *Bact. coli* which, by attenuation, has lost its power to produce indole, whereas the same organism, when isolated from human excrement, does not fit into the classification of faecal coliforms. Any division of the coliform bacteria into faecal and non-faecal types must, however, depend not only upon biological reactions, but upon the frequency with which each type can be isolated from the intestines of warm-blooded animals, making due allowance for the undoubted fact that typically non-faecal coliforms may be found in excrement through accidental ingestion with foods, and typically faecal coliforms are frequently found outside the intestine as a result of contamination with excrement.

Table 4 shows coliform types from filtered and chlorinated water and how seldom Irregular Type II is isolated. Its classification is, therefore, of academic interest rather than of practical importance in water analysis.

Table 4. (*a*) *Frequency of isolation of coliform types from filtered and chlorinated water*

Number of samples	15,770
Number containing coliforms	425 (2·7 %)
Number containing *Bact. coli* Type I	225 (1·4 %)

(*b*) *Classification of the coliform types isolated*

Coliform types isolated:	425
Bact. coli Type I	225 (52·9 %)
Irregular Type I	10 (2·4 %)
Irregular Type II	4 (0·9 %)
Bact. coli Type II	5 (1·2 %)
Intermediates	86 (20·2 %)
Bact. aerogenes	62 (14·6 %)
Irregular Type VI	33 (7·8 %)

Irregular Type VI. The other coliform organism which gives a strong reaction at 44° has otherwise all the attributes of *Bact. aerogenes*, namely, acid and gas in lactose, indole negative, growth in citrate, methyl red negative, Voges-Proskauer positive.

This organism is rarely found in faeces in England, occurring only twice in the series of 780 examinations mentioned above. It is most frequently isolated from water samples collected from new or repaired water mains and wells (Whiskin & Taylor, 1945). The source in these cases has been traced to jute yarn used as a jointing material in mains and as a packing in well pumps. For this reason it has been named the 'yarn' organism, but is Irregular

Type VI in Wilson's classification of coliforms. It has also been isolated from decaying wood; for example, a coliform count of 16/ml. was obtained from a sample of well water which had been negative for coliform bacteria for some months previously. All primary tubes gave a positive confirmatory 44° test and Irregular Type VI was isolated in pure culture. Investigation ultimately traced the origin of the infection to decaying wood in the adits of the well, from which material the type was freely cultured.

Irregular Type VI is much more common in India, being isolated frequently from stools and for that reason the 44° test has not gained favour there (Raghavachari & Iyer, 1939; Boizot, 1941). Information kindly supplied by dealers in yarn packing is to the effect that much of the jute fibre is imported from Calcutta and is then spun and made up in this country. Irregular Type VI is constantly found in this material and it seems highly probable that these organisms are imported in the jute, and that there is sufficient moisture on the fibre to keep the organisms alive. They survive the manufacture into yarn and, under the favourable temperature conditions ruling during the summer months in this country, the organisms multiply in the yarn and on appearing in water samples are responsible for failures of the 44° test as an indicator of *Bact. coli* Type I similar to those reported from India.

This coliform organism is capable of surviving for long periods and multiplying in jute, and the use in waterworks of jute yarn or other materials infected with it is to be deprecated, otherwise water supplies are likely to be condemned owing to its presence. When Irregular Type VI is suspected as being the cause of a positive reaction to the 44° test it is necessary to plate out and type the organism fully before an opinion can be given on the safety of the water. This procedure requires 5 days and causes much unnecessary delay.

The differential value of indole production at 44°

Production of indole is characteristic of *Bact. coli* Type I and is shared only by four other coliform organisms, namely, Irregular Types I and VII, Intermediate Type II and *Bact. aerogenes* Type II. On the other hand, these organisms differ from *Bact. coli* Type I in that they are incapable of fermenting lactose at 44°. Again, of the three coliform organisms that are able to ferment lactose at 44°, only one, namely *Bact. coli* Type I, produces indole. It therefore appeared that, if *Bact. coli* Type I were capable of indole production at 44° in pure and in mixed culture, then a rapid mode of differentiation was available.

Accordingly several cultures were tested for indole production in the water-bath at 44° (Table 5).

All *Bact. coli* Type I strains produced indole at 44°, and all intermediate and aerogenes strains failed to do so. Six of the seventeen Irregular Type I strains produced indole at 44°, and it is known that four of these were isolated from stools. Thus, by inoculating from a primary positive tube into brilliant green bile broth and a tube of peptone water for indole production and incubating both at 44° for 24 hr., the resulting reactions would indicate *Bact. coli* Type I, or other coliform bacteria as shown in Table 6.

An anomalous result would occur if there were a mixture of Irregular Type VI and certain strains of Irregular Type I. Such a combination, however, would be extremely rare.

In an endeavour to ascertain whether small numbers of *Bact. coli* can be detected by indole formation at 44°, even when growing in competition with Irregular Type VI, a few experiments were devised of which the following is an example. A series of seven tubes of brilliant green bile broth and seven of peptone water were each inoculated with about sixteen viable *Bact. coli* Type I and with a volume of suspension of Irregular Type VI sufficient to give a concentration in the different tubes varying from 150 to 150,000 organisms. The numbers of organisms in the inocula were estimated by MacConkey plate counts. Following 24 hr. incubation at 44° all the peptone water tubes were positive by the indole test and all brilliant green bile broth tubes showed gas production. It was to be expected that the brilliant green bile broth tubes would develop gas with either organism but the presence of *Bact. coli* Type I was demonstrated by the indole test even in as low an initial ratio as about 1 : 10,000.

Table 5. *Indole production at 44° by various coliform types*

Coliform type	No. of strains	Strains producing indole at		Strains producing gas in brilliant green bile broth at 44°
		37°	44°	
Bact. coli Type I	59	59	59	59
Irregular Type I	17	17	6	0
Intermediate Type II	12	12	0	0
Bact. aerogenes Type II	19	19	0	0

Table 6. *Differentiation of coliform types by gas production in brilliant green bile broth at 44° and indole production at 44°*

Gas in brilliant green bile broth at 44°	Indole production at 44°	
+	+	*Bact. coli* Type I
+	0	Irregular Type II Irregular Type VI
0	+	Other coliforms
0	0	Other coliforms

This experiment indicates the sensitivity of the 44° indole test for *Bact. coli* Type I even in the presence of relatively large numbers of non-typical coliform organisms.

CONCLUSIONS

A simple procedure has been perfected to differentiate Irregular Type II and Irregular Type VI from *Bact. coli* Type I. A tube of brilliant green bile broth and a tube of peptone water are inoculated from a positive MacConkey broth tube. Both tubes are then incubated for 24 hr. in a specially controlled 44° water-bath. At the end of this time the brilliant green bile broth tube is read for gas production and the peptone water tube is tested for indole. A positive result from each test indicates the presence of *Bact. coli* Type I.

This modified version of the 44° test for the rapid identification of *Bact. coli* Type I has several advantages over existing tests. It is, for all practical purposes, specific for *Bact. coli* Type I. It enables the water bacteriologist to give an accurate estimate of the *Bact. coli* content of water within 2 days of drawing a sample as against 5 days by the plating method. There is also a saving in media, apparatus and labour required for isolation and differential tests. The time saved makes it possible to examine more samples each day so that water supplies and treatment plants can be more closely supervised and the purity of the supply more efficiently controlled.

The application of the method, outlined above, to water samples in the Tropics would be worthy of trial in view of adverse reports on the 44° fermentation test emanating from certain eastern countries.

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The Influence of Antibacterial Substances on the Interaction of Bacteria and Bacteriophages

1. The Influence of Penicillin

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SUMMARY: Penicillin in concentrations up to 100 units/ml. in broth or synthetic media has no demonstrable effect, after 20 hr. incubation at 37°, on the activities of Staphylococcus K phage, Coli-phage C 36, Coli-dysentery phage S13, a Streptococcal phage and a *Bacillus subtilis* phage.

The simultaneous action of penicillin and phage on young cultures of *Staphylococcus aureus* (Oxford) in broth or synthetic medium at 37° produces, under certain conditions, a more rapid lysis than occurs in the presence of penicillin or phage alone.

The phenomenon of accelerated lysis through the joint action of penicillin and phage occurs with other organisms besides *Staph. aureus*, e.g. *B. subtilis* and *Streptococcus pyogenes*, Group C, differing from that with *Staph. aureus* only in degree.

Penicillin does not affect the adsorption of phage by the organisms. When the amount of antibiotic is sufficient to interfere adversely with the growth of the cell then the multiplication of phage decreases. It is suggested that certain balanced intracellular reactions of metabolism are disturbed by the action of penicillin, and as a result, intermediates essential to growth both of cell and phage cease to be available.

A phage-inhibiting substance was demonstrable in certain instances when *Staph. aureus* (Oxford) cultures were lysed by penicillin.

Bacteriophages, or bacterial viruses, as they are now frequently called, are parasites requiring the environment prevailing within the actively multiplying host cell for their continued propagation. There are certain well-defined chemicals and also substances of biological origin, antibiotics, whose chemical structure is not in every case known, but all of which, in varying degree, interfere adversely with the normal metabolism of the bacterial cell, arresting growth and often causing death. Studies of the combined action of such antibacterial agents and phages on bacteria, therefore, may furnish evidence as to whether a particular substance interferes with the intracellular reactions prerequisite for the multiplication of the respective bacteriophages. The chemical nature of the interfering substances being known, some indication may be gleaned of the type of reaction involved, and of the underlying processes determining both bacterial and virus multiplication. It was with such possibilities in mind that the present studies were initiated.

Its effectiveness as an antibiotic against a wide range of organisms made penicillin an obvious choice for early investigation. It is now available in highly purified form, notably as the crystalline sodium or calcium salt (Report, 1945). The present paper describes a detailed study of the influence of penicillin upon the interaction of *Staphylococcus aureus* and an anti-staphylococcus phage. Its influence on some other organisms, and their respective phages has been studied, but in less detail.

MATERIALS AND METHODS

Organism. The Oxford 'H' strain of *Staph. aureus*, widely employed in penicillin studies in recent years, has been used, and the S3K strain originally obtained from Dr A. P. Krueger of the University of California. Both strains are readily susceptible to Staph. K phage, but the results recorded here refer to the Oxford strain unless otherwise stated. The culture media were papain broth, tryptic digest broth (Hartley, 1922) and the defined medium of Fildes & Richardson (1937). The growth curves have been determined both by the plate count of viable organisms and by the combined visual and photoelectric estimation of the turbidities of cultures.

Bacteriophage. The anti-staphylococcus bacteriophage Staph. K, also originally obtained from Dr Krueger, has been used throughout. Determination of phage concentrations have been made by the plaque-count method, employing 1 % nutrient agar.

Penicillin. I am much indebted to my colleague, Mr P. Bruce White, for furnishing accurately standardized solutions of penicillin in phosphate buffer at pH 7. The concentrations of penicillin are expressed in terms of international units/ml. The early experiments were made with solutions of the calcium salt from commercial preparations of comparatively low grade, 150 u./mg. Later, solutions of the crystallized sodium salt of penicillin G—potency 1600 u./mg.—were used. The data presented, unless otherwise stated, are for the sodium salt. The solutions were sterilized by filtration through gradocol membranes of porosity 0·4–0·5 μ. Penicillin solutions may be filtered through such membranes without detectable loss of activity.

Abbreviations used. Staph. O = *Staphylococcus aureus*, Oxford strain; $[S]_0$ = initial concentration of staphylococci in organisms/ml.; P = penicillin; $[P]$ = concentration of penicillin u./ml.; $[\phi]_0$ = initial concentration of bacteriophage in particles/ml.; ϕ = bacteriophage; ϕI = phage-inhibitor.

THE ACTION OF PENICILLIN AND PHAGE ON *STAPHYLOCOCCUS AUREUS*

The action of penicillin on Staph. O

Fleming (1929), in his original paper on the effect of penicillin on a culture of *Staph. aureus*, states 'the staphylococcus colonies became transparent and were obviously undergoing lysis'. This observation has since been supplemented by numerous studies of the action of penicillin under varied conditions, and many of the published facts have been confirmed in the course of the present work. All experiments were made with the staphylococcus culture in its logarithmic phase of growth, since not only is this most favourable for the bacteriophage but penicillin also has been found to exert its action most strikingly on the multiplying organism (Bigger, 1944; Hobby & Dawson, 1944*a*, *b*; Todd, 1945*a*; Fisher, 1946). On the addition of an appropriate amount, say 0·1 u./ml. of penicillin, to such a culture the organisms appear to multiply normally for a short period; then there is a decrease in the rate of increase of viable organism, soon followed by a decrease in the viable count, which falls off rapidly. The turbidity increases less rapidly soon after the viable count has begun to fall, and finally decreases as lysis commences. This continues until the culture is relatively clear, only opalescent. The quantitative aspect of the action of penicillin in relation to its concentration is clearly shown in Fig. 3. The findings confirm those of Rantz & Kirby (1944), Nitti, Fossaert & Faguet (1944), Lee, Foley & Epstein (1944), and Todd (1945*a*), amongst others. Optical studies with the ultra-violet light microscope and also the electron microscope, supplementing the observations recorded in this paper (Smiles, Welch & Elford, 1948), conclusively demonstrate, in agreement with Gardner (1940), Smith & Hay (1942) and Fisher (1946), that the organism quickly swells in the presence of penicillin to almost twice its normal size before lysis.

The action of phage on Staph. O

Staph. aureus 'Oxford' was more readily adapted to grow in synthetic medium than the S3K strain. The course of phage action on this organism was very similar to that observed on S3K, the cultures in broth and synthetic media being lysed completely. The phage titres of such lysed cultures were invariably of the order 5×10^9 particles/ml.

The action of penicillin on Staph. K phage

Known concentrations of penicillin in (*a*) 0·9 ml. papain broth, and (*b*) 0·9 ml. defined medium, each received 0·1 ml. of a 1/100 dilution, in the corresponding medium, of Staph. K phage (0·7 μ. membrane filtrate of a lysed culture of Staph. O). The appropriate controls without penicillin were included, and all systems, in $3 \times \frac{3}{8}$ in. tubes with rubber caps, were incubated for 20 hr. at 37°. Then 1/10,000 dilutions were made in saline broth and plated in triplicate with Staph. O. The results of a number of such experiments showed that penicillin, in concentrations up to 100 u./ml. in broth or synthetic medium, and in one test up to 400 u./ml. in broth, was without significant effect on Staph. K phage. This agrees with the findings of Neter & Clark (1944), Himmelweit (1945), Jones (1945), Nicolle & Faguet (1947) and Rountree (1947).

The combined action of penicillin and phage on Staph. O

The first series of experiments made in 1944 demonstrated that young cultures of Staph. O, in broth or synthetic medium, were lysed more rapidly by the combined action of penicillin and phage than were control cultures containing either penicillin or phage alone. The observations in a typical experiment are given in Table 1. The accelerated lysis was so pronounced that a detailed study was undertaken. Meanwhile, similar observations were independently recorded by Himmelweit (1945), and confirmed and extended by Nicolle & Faguet (1947).

(i) *The effect of time of contact between Staph. O and penicillin before the addition of phage, on the times of lysis and yield of phage.* Five ml. amounts of

Staph. O culture in the logarithmic phase of growth in papain broth at 37° were inoculated with 0·1 ml. penicillin solution to give $[P]=0{\cdot}1$. After chosen periods of incubation 0·1 ml. phage filtrate was added to each system as indicated in Table 2, which gives the phage concentrations after 1, 3 and 24 hr. Fig. 1 presents the turbidity data from photoelectric measurements in a similar experiment. Clearly, the phenomenon of accelerated lysis by $P+\phi$, although still present, becomes less pronounced as the initial contact with P is extended. The yield of phage decreases progressively with the time of exposure of Staph. O to penicillin. It is also of importance to note the maximum level of turbidity reached by the various systems before lysis sets in.

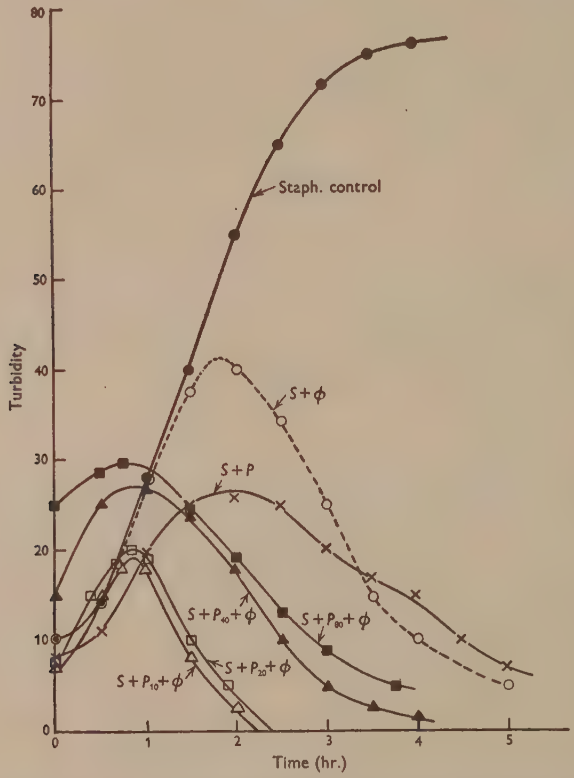

Fig. 1. Turbidity/time curves for *Staph. aureus* broth cultures inoculated with Staph. K phage after different periods of treatment with 0·1 u./ml. penicillin at 37°. $[\text{Staph.}]_0=4{\cdot}4\times10^7$/ml. $[\phi]_0=1{\cdot}5\times10^7$/ml. $P_{10,\,20,\,40,\,80}$ = time (min.) culture had been incubated with penicillin before infection by phage.

(ii) *The action of phage on a penicillin-resistant variant of Staph. O.* A penicillin-resistant (P.R.) variant of Staph. O was trained to grow in the presence of 40 u./ml. penicillin by Mr J. Bligh. This resistant variant grew more slowly and the colonies on agar were smaller and less opaque than those

Table 1. *The combined action of* $1\cdot4\times10^7$ *phage particles/ml. and varying quantities of penicillin on Staph. O in papain broth at* 37°

Staph. O ($[S]_0=4\cdot8\times10^7$) treated with	Turbidities at times (min.)												
	0	15	35	50	70	80	90	100	113	130	160	180	(21 hr.)
Nil	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
ϕ	+	++	+++	+++	+++	+++	+++	+++	+++	++	+	–	–
$[P]=10$	+	++	+++	+++	++	++	++	+	+	+	+	+	–
$[P]=10+\phi$	+	++	+++	+++	–	–	–	–	–	–	–	–	–
$[P]=1$	+	++	+++	+++	++	++	++	+	+	+	+	+	–
$[P]=1+\phi$	+	++	+++	+++	–	–	–	–	–	–	–	–	–
$[P]=0\cdot1$	+	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	±
$[P]=0\cdot1+\phi$	+	++	+++	+++	+	±	–	–	–	–	–	–	–
$[P]=0\cdot01$	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
$[P]=0\cdot01+\phi$	+	++	+++	+++	+++	+++	+++	+++	++	++	–	–	–

±, +, ++, +++ = slight to maximum turbidities of culture; – = no turbidity.

Table 2. *Influence of time of contact between Staph. O* ($[S]_0=7\cdot9\times10^7$) *and penicillin* ($[P]=0\cdot1$) *before addition of phage* ($[\phi]_0=2\cdot5\times10^7$), *on yields of phage*

Time of contact of Staph. O with penicillin before adding phage (min.)	Phage particles/ml. after		
	1 hr.	3 hr.	24 hr.
0	$3\cdot9\times10^8$	$3\cdot1\times10^9$	$2\cdot5\times10^9$
5	$2\cdot3\times10^8$	$6\cdot7\times10^7$	$4\cdot5\times10^7$
10	$1\cdot3\times10^8$	$5\cdot6\times10^7$	$1\cdot7\times10^7$
20	$1\cdot08\times10^8$	$1\cdot8\times10^7$	$3\cdot1\times10^6$
40	$9\cdot4\times10^7$	$4\cdot8\times10^6$	$1\cdot2\times10^6$

of the normal sensitive organism. A broth culture of this P.R. variant was completely lysed by Staph. K phage at 37°. Demerec (1945) has reported a similar finding in his experiments with penicillin-resistant mutants of *Staph. aureus*. When our phage was titrated against P.R. Staph. on 1% agar the plaques were definitely larger (0·5–1 mm.) than those usually developed against Staph. O (0·1–0·25 mm.). The P.R. Staph. would appear to be abnormally sensitive to the phage, but the more probable explanation lies in the rate of propagation of the phage relative to the leisurely growth characteristic of this P.R. variant (cf. the interpretation of influence of penicillin on plaque size in next paragraph).

(iii) *Phage plated with staphylococci on agar containing penicillin.* Staph. K phage was plated with Staph. O on 1% nutrient agar containing known concentrations of penicillin. $[P]=0{\cdot}1$ completely inhibited the organism. The combined action of phage and penicillin resulted, under certain conditions—$[P]=0{\cdot}01$—in the formation of larger plaques, 0·5–1·0 mm., than normally observed. It appears that, as observed in broth systems, penicillin retards the active growth of the staphylococci, and in effect, enhances the zone of influence of each phage particle so that cells become infected and lysed over a greater area before their age and concentration arrest the process.

(iv) *The influence of the initial concentration of staphylococci on the outcome of the combined action of penicillin and phage.* Staph. O in the logarithmic phase of growth in papain broth at 37° was diluted to give a serial range of concentrations 10^5–10^9/ml. Four experimental systems were prepared for each $[S]_0$; $[\phi]_0=3\times10^7$ and $[P]=1$ in all cases. The results are shown graphically in Fig. 2. This type of experiment provided much information. First, there is the quantitative aspect of the action of a given $[P]$ on different $[S]_0$. Secondly, the yield of phage from a standard $[\phi]_0$ in relation to various $[S]_0$ is shown, and thirdly, our chief interest, the yield of phage and the incidence of lysis when the same $[P]$ and $[\phi]$ act together on different $[S]_0$.

When $[S]_0$ is 10^9 the growth is poor; presumably competition for metabolites is so great that the organisms are unable to multiply many times. When $[P]=1$ there is only a slight decrease in viable count, suggesting that either the antibiotic is deterred through the sluggish growth of the organisms, or, that the amount of penicillin is inadequate. The curves for the growth of Staph. O alone and for Staph. O plus penicillin demonstrate very clearly that penicillin acts with greatest effect on cultures in which the organisms are growing freely. Conditions most favourable for the multiplication of phage obtain when the number of actively growing organisms is greatest. The multiplication of phage in the presence of penicillin is diminished according to the extent to which the antibiotic has interfered with the activity of the organisms. The form of the curves showing the falling off of phage activity with time suggests that a phage-inhibiting substance may be present (see below).

In summary, therefore, the degree in which $[S]_0$ affects the outcome of the combined action of P and ϕ is a function of the vitality of the culture within the first few hours of incubation. Under the conditions of our experiment, i.e. for $[P]=1$ and $[\phi]_0=3\times10^7$, lysis is accelerated most in the zone $[S]_0=10^7$–10^8.

It may be noted that in these conditions each organism would be infected by a single particle (Delbrück & Luria, 1942). In more concentrated cultures the growth of Staph. O is less prolific and in consequence the lytic potency of P and ϕ is reduced, whereas for the lower values of $[S]_0$, when multiple infection by ϕ can occur, all sensitive organisms are rapidly eliminated even by ϕ alone.

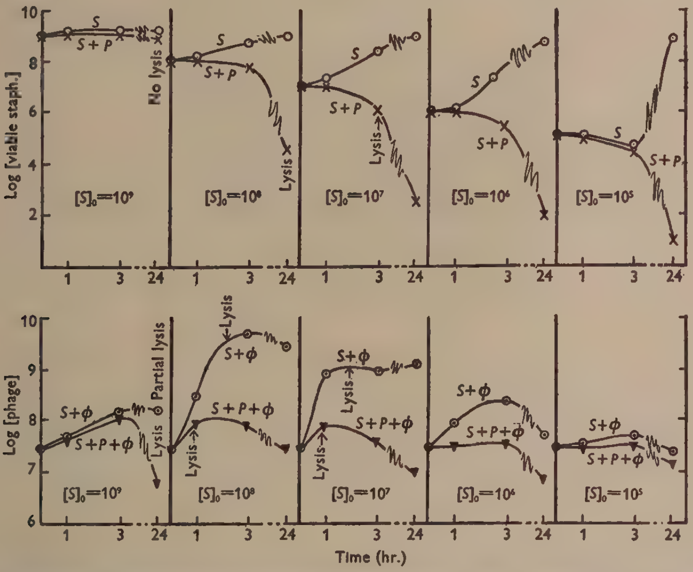

Fig. 2. Influence of initial concentration of *Staph. aureus* on the outcome of the interaction with penicillin and phage. ○ = Staph. control, × = Staph. + penicillin, ⊙ = Staph. + phage, ▼ = Staph. + phage + penicillin. $[P]=1$ u./ml. $[\phi]_0=3\times10^7$/ml.

(v) *The influence of the concentration of penicillin.* The influence of $[P]$ has been investigated in both broth and defined medium with similar results. The curves in Fig. 3 show how, for a given $[S]_0$ and $[\phi]_0$, the development of the culture can be influenced by the presence of penicillin in concentrations ranging from 40 to 0·0004 u./ml. Little growth of the staphylococci occurs when $[P]=0{\cdot}4$ to 40 and visible lysis sets in sooner than in the control (Staph. O + P) or (Staph. O + ϕ) systems. The phage titre increases slightly during the first hour, but, thereafter, decreases as the action of penicillin combines to lyse the organisms. A notable increase in the development of phage occurs at $[P]=0{\cdot}04$, where the initial slope of the Staph. O growth curve approximates that of the control. However, it is not until the growth curve of the organism approaches even more closely that of the control that the yield of phage approaches that in the culture containing no penicillin. Even with $[P]$ as low as 0·0004 a broth culture of staphylococci is clearer in 4 hr. than in the absence of penicillin, although otherwise the curves are much alike.

Another interesting point concerns the shape of the curve showing the variation of ϕ concentration with time. There is a definite maximum after about 1 hr. for $[P]=0{\cdot}4$–40, but at $[P]=0{\cdot}04$ the maximum is much later and less pronounced, while for yet smaller $[P]$ the curve becomes very like the control where $[P]=0$. This falling off in phage titre in the presence of penicillin (see also Tables 2 and 3) may be explained in one of two ways: (*a*) the lysis of the organisms may be accompanied by the liberation of a phage-inhibiting substance (ϕI); or (*b*) phage becomes irreversibly adsorbed to cellular debris and so is not available for infecting susceptible organisms. Early experiments

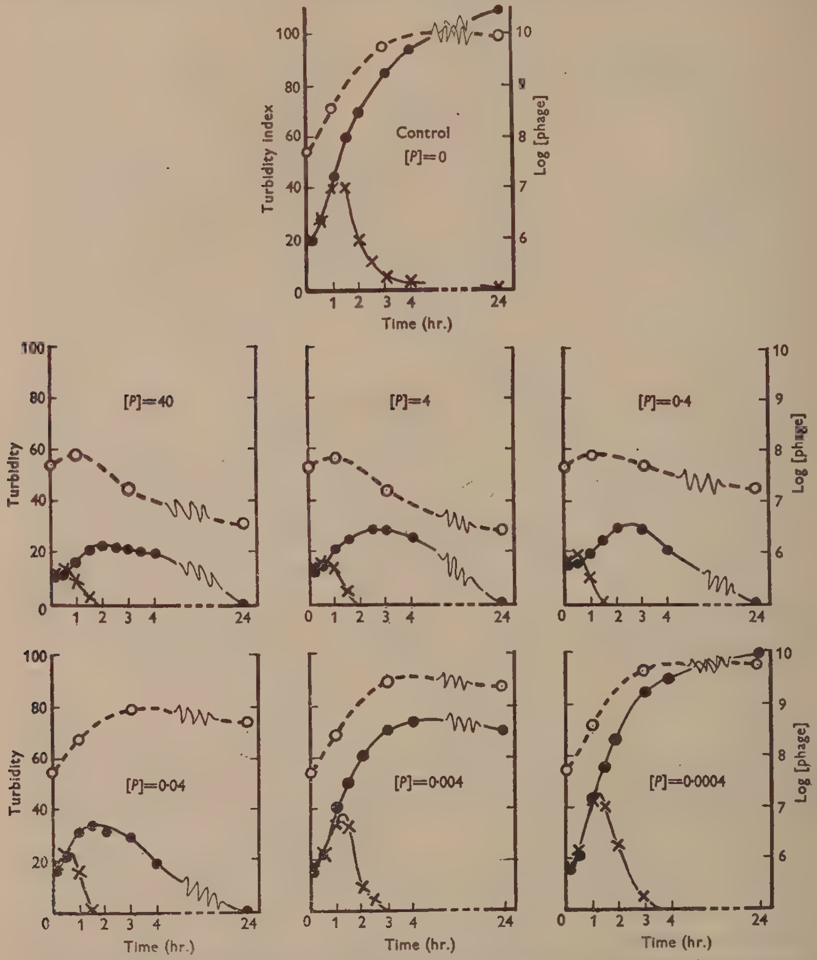

Fig. 8. Influence of penicillin concentration on the interaction of penicillin and phage on *Staph. aureus*. ○ = concentration of phage, ● = turbidity of $S+P$, × = turbidity of $S+P+\phi$. $[\text{Staph.}]_0=6{\cdot}5\times10^7$/ml. $[\phi]_0=4{\cdot}9\times10^7$/ml.

with a low-grade penicillin gave evidence favouring the former of these interpretations, since the presence of ϕI was readily demonstrated in 0·7 μ. membrane filtrates of the staphylococcus culture lysed by penicillin. The filtration end-point for ϕI was close to that of the phage itself—namely 100 mμ. It was found, furthermore, that the ϕI could be precipitated from the filtrate at 0° by adding 60 % ethanol, and this provided a means for its partial purification and concentration. A preparation concentrated in this way showed an interesting dilution phenomenon. The undiluted fluid exhibited only a slight ϕ-inhibiting power, but when diluted 10- or 100-fold it proved strongly inhibiting for ϕ, suggesting that the medium might contain a substance which blocked the action of ϕI substance. This was found to be the case, since by treating the papain broth with 60 % alcohol a precipitate was formed, which, when taken up in water was found to possess the property of blocking the action of ϕI. In experiments, however, with purified penicillin acting on the staphylococci in papain broth or defined medium, though the culture was lysed, no clear evidence of the presence of ϕI in 0·7 μ. membrane filtrates could be obtained, although the opalescent unfiltered liquid was strongly inhibitory. Two possible explanations of this change in behaviour were considered. The low-grade penicillin might have contained as an impurity an enzyme which, in combination with penicillin, facilitated the more complete disintegration of the cell structure than can be achieved by penicillin alone. Unfortunately, the batch of penicillin with which the early experiments were made was exhausted before the matter could be thoroughly investigated. An alternative explanation may be sought in mutation of the organism yielding a variant with altered susceptibility to penicillin. Evidence that mutation can occur has been met twice during the studies with Staph. O in broth and synthetic medium, colonies manifested by their subnormal size (s.c.) accompanying normal-sized colonies. On subculturing, the s.c. variant was found to be more sensitive to penicillin and also yielded larger plaques with Staph. K phage—1 mm. as against the usual 0·25 mm. This variant reverted after a period to the normal colony type, and the phage plaques likewise reverted to normal small size. The s.c. variant lysed by penicillin and then filtered through 0·7 μ. membrane yielded a filtrate that reduced the ϕ-titre by 75 % during overnight incubation at 37°. A phage-inhibiting substance has been extracted from autolysed staphylococci by Levine & Frisch (1932) and Gough & Burnet (1934), and also from staphy-

Table 3. *The multiplication of phage in Staph. O cultures* ($[S]_0 = 8 \times 10^8$) *with and without* 1 *u./ml. penicillin, for various values of* $[\phi]_0$.

	Ratio of final to initial concentrations of phage after			
	3 hr.		24 hr.	
$[\phi]_0$	Without penicillin	With penicillin	Without penicillin	With penicillin
3×10^3	28	7	33,000	13
3×10^5	50	10	730	5
3×10^7	32	6	13	4·7
3×10^9	3	1·8	1·9	0·4

lococci digested with trypsin by Freeman (1937). Burnet & Gough concluded that the inhibiting substance was a complex polysaccharide. It is interesting that filtration indicated the size of the inhibiting substance liberated by penicillin to be of the same order as that of the phage itself.

(vi) *The influence of the initial concentrations of phage.* The phenomenon of accelerated lysis of Staph. O cultures through the joint action of penicillin and bacteriophage was most clearly manifested within a range of $[\phi]_0 = 10^7$ to 10^9 when $[S]_0 = 10^7$ to 10^8, i.e. for single particle infection of organism by phage. Where multiple infection could occur, $[\phi]_0 > 10^9$, the times of lysis with and without penicillin did not differ significantly. On the other side of the optimum zone the phage can infect initially only a fraction of all organisms, and hence lysis is much delayed.

The profound effect of penicillin on the multiplication of the phage is well illustrated by the ratio of the concentration of phage after 3 and 24 hr. to the initial phage concentration (Table 3). It is also interesting to note that in addition to the inhibition of phage following lysis of staphylococci when penicillin is present, so also, for high concentrations of phage alone, when lysis is complete, partial inactivation of phage can apparently occur.

THE EFFECT OF PENICILLIN ON THE ADSORPTION OF PHAGE ON STAPHYLOCOCCI

Adsorption experiments were made in Hartley's broth and defined medium, and in each instance there was no significant difference between the amount of phage adsorbed by normal staphylococci and organisms that had been exposed to the action of penicillin in concentrations up to 5 u./ml. for periods up to 2 hr. It is unlikely, therefore, that penicillin interferes with the initial step in process of infection of staphylococci by phage. Rountree (1947) found that staphylococci after 3 hr. contact with 5 u./ml. penicillin at 37° but not lysed by it, adsorb phage as readily as normal staphylococci.

Inactivation of penicillin by cysteine

It was confirmed that cysteine in appropriate concentration inactivates penicillin at pH 6·8–7·0. Penicillin so treated no longer affects the lysis of staphylococcal cultures in the presence of phage.

The filterability of Staph. K phage from culture lysed by the joint action of penicillin and phage

The filtration end-point for phage from Staph. O culture lysed by penicillin plus phage was the same as for phage propagated in absence of penicillin, namely 100 mμ.

The effect of penicillin on burst size

The experimental procedure described by Delbrück & Luria (1942) was used to ascertain whether penicillin has any effect on (*a*) the latent period elapsing between the moment of infection and the liberation of phage following lysis of the organism; and (*b*) the 'burst size' or, the average number of phage particles liberated per infected cell. The results are summarized in Table 4, and some typical step-wise growth curves are given in Fig. 4. It will be seen that when $[P]=0{\cdot}1$ or less, in a concentration known to be definitely inhibitory for the continued growth of the staphylococci, the process of infection and phage multiplication appears to proceed with little if any departure from the normal course. The first abnormality noted as $[P]$ is increased is the belated onset of the second step-wise increment in phage concentration and its eventual elimination. Ultimately, the average yield of phage per infected cell is reduced, but the latent period is not appreciably affected. Clearly, if all the cells are infected initially their lysis would be expected to occur simultaneously in (Staph. O $+\phi$) and (Staph. O $+P+\phi$) systems. This has been observed to be so when conditions of multiple infection prevail. The general effect of penicillin on the course of phage action is the same in broth and synthetic medium. The mean generation time of Staph. O in defined medium was 40–45 min.

Table 4. *Burst sizes for Staph. O lysed by Staph. K ϕ with and without penicillin*

Medium	Latent period (min.)	Burst size	$[P]$	$[S]_0$	$[\phi]_0$	Remarks
Hartley's broth	39	47	—	2×10^8	$1{\cdot}2\times10^7$	2nd rise after 36 min.
Hartley's broth	38	52	0·01	2×10^8	$1{\cdot}2\times10^7$	2nd rise after 31 min.
Hartley's broth	38	54	—	$2{\cdot}75\times10^8$	$1{\cdot}1\times10^7$	2nd rise after 33 min.
Hartley's broth	35	49	0·1	$2{\cdot}75\times10^8$	$1{\cdot}1\times10^7$	No 2nd rise in 1½ hr.
Hartley's broth	38	45	—	$2{\cdot}3\times10^8$	$9{\cdot}2\times10^6$	2nd rise after 33 min.
Hartley's broth*	34	47	0·1	$2{\cdot}3\times10^8$	$9{\cdot}2\times10^6$	Gradual rise after 40 min.
Hartley's broth	40	40	—	$1{\cdot}4\times10^8$	$9{\cdot}2\times10^6$	2nd rise after 24 min.
Hartley's broth*	37	21	2·0	$1{\cdot}4\times10^8$	$9{\cdot}2\times10^6$	No 2nd rise in 2 hr.
Synthetic	65	25	—	5×10^8	$1{\cdot}8\times10^7$	2nd rise not investigated
Synthetic	65	28	0·2	5×10^8	$1{\cdot}8\times10^7$	—
Synthetic	60	15	0·5	$2{\cdot}6\times10^8$	$1{\cdot}8\times10^7$	No 2nd rise in 4 hr.
Synthetic	60	24	—	$5{\cdot}8\times10^8$	$1{\cdot}8\times10^7$	2nd rise after 180 min.

* Penicillin present during initial adsorption.

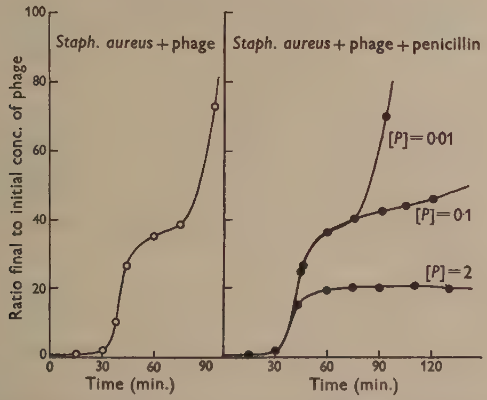

Fig. 4. Step-wise growth curves for Staph. K phage on *Staph. aureus* in broth, with and without penicillin.

compared with 22–25 min. in papain broth. The corresponding latent periods of phage multiplication were 60–65 min. and 35–38 min., and the 'burst sizes' 25 and 48 respectively.

THE INFLUENCE OF PENICILLIN AND PHAGE ON ORGANISMS OTHER THAN *STAPHYLOCOCCUS AUREUS*

The action of penicillin on other bacteriophages. Tests similar to those described for Staph. K phage were made with coliphage C36, a coli-dysentery phage S13, a subtilis phage and a streptococcus phage. Concentrations of penicillin up to 100 u./ml. in broth medium were without significant action on these bacteriophages, after 20 hr. at 37°.

The combined action of penicillin and phage

Bacillus subtilis. The strain of *B. subtilis* and its phage were received through the kindness of Dr Wahl of the Pasteur Institute, Paris. The phenomenon of accelerated lysis was readily demonstrable in young broth cultures of *B. subtilis* at 37°, infected by phage in the presence of 10 u./ml. penicillin.

Bact. coli. The strain of *Bact. coli* used was partially inhibited when penicillin (100 u./ml.) was added to a young broth culture at 37°. The onset of lysis in the presence of phage C36 and penicillin 100 u./ml. was only very slightly earlier than that occurring with phage alone. The quantitative estimation of viable organisms and phage confirmed that the penicillin was exerting a definite antibacterial effect.

Shigella flexneri Y6R (National Collection of Type Cultures). Penicillin definitely inhibited *Shig. flexneri* Y6R growing logarithmically in broth at 37° when the concentration of antibiotic reached 100 u./ml. With $[P]=100$ and S13 phage there was no evidence of accelerated lysis. However, the yield of phage was low, undoubtedly a consequence of the antibiotic action exerted by the penicillin.

Streptococcus pyogenes, Group C, type 7. Streptococcus C, type 7, growing actively in yeast-Lemco-broth at 37°, was inhibited by 0·02 u./ml. penicillin. Accelerated lysis by penicillin plus phage was readily demonstrated. Further, the onset of lysis with $(P+\phi)$ was strikingly sharp. The multiplication of ϕ was decidedly less in the presence of penicillin than in the control.

Thus, though the phenomenon of accelerated lysis by penicillin and phage appeared to be general for organisms readily susceptible to the antibiotic, there were differences of degree according to the organisms concerned.

DISCUSSION

The most generally accepted view is that penicillin exerts its antibiotic action by interfering, directly or indirectly, with some essential reaction in the metabolism of the cell. Several writers have suggested that penicillin modifies the permeability of the cell wall, and, in consequence, the assimilation of essential growth factors (Smith & Hay, 1942). Recently Gale & Taylor (1946) have shown that penicillin does influence the assimilation of glutamic acid by

Staphylococcus aureus. The upset of oxidation-reduction balance within the cell has been considered by others to be the basis of the mode of action of penicillin (Mulè, 1946; Krampitz, Green & Werkman, 1947; Dufrenoy & Pratt, 1947). The course of action of penicillin proceeds thus—arresting of growth → loss of viability → final lysis. The lytic phase has been attributed by Todd (1945*b*) and Fisher (1946) to the action of bacterial autolysin, which, when growth ceases, becomes exceptionally active. Now that our knowledge of the chemical nature of penicillin is fairly complete, extended studies of the type of reaction into which it can enter (e.g. interference with normal function of —SH groups) should eventually throw light on its interaction with the cell. In contrast to penicillin, which is a relatively simple substance chemically, bacteriophage is much larger and more complex. The few phages so far submitted to analysis have been found to be nucleoproteins. Essentially a bacterial parasite, the phage finds conditions for its growth satisfied by the intracellular environment, but the precise requirements have not yet been elucidated. Some intermediate in the cell's synthetic processes (nucleic acid metabolism?) may be also a substrate for which the phage actively competes. As a result of infection by phage the cell generally succumbs and is finally lysed.

When a young culture of *Staph. aureus* is attacked simultaneously by penicillin and Staph. K bacteriophage, it is lysed more rapidly than when penicillin or phage acts alone. Certain conditions of infection are needed to ensure the ready demonstration of this striking effect. The concentration of the young culture should be 10^7–10^8 organisms/ml. and the initial concentration of phage such that most of the organisms become infected. When $[\phi]_0 \gg [S]_0$ then almost all the cells will be infected by at least one phage particle and no appreciable acceleration of lysis is observed. If, however, the clearing of the culture is dependent on at least two cycles of infection then the accelerated lysis by $P+\phi$ is readily demonstrated. This suggests that the staphylococci which have been exposed to the action of penicillin are more easily lysed by phage than normally. A phage-free ultra-filtrate of a lysed Staph. O culture does not accelerate lysis of Staph. O in the presence of penicillin; neither does heat-inactivated phage. The accelerated lysis is, therefore, the outcome of the combined action of intact phage and penicillin. Detailed analysis of this joint action has shown that:

(i) The presence of penicillin, in concentrations far in excess of that inhibitory to cell growth and acting for periods longer than the mean generation time, does not influence the adsorption of phage by the cell. This indicates that the phage receptor groups are not affected, and any modification in the course of infection should be looked for in an altered rate of penetration and possible changes in the intracellular processes.

(ii) When penicillin has seriously affected the growth of the cell then the yield of phage falls. This supports the hypothesis that phage is dependent upon some intracellular intermediate as substrate in its own multiplication.

(iii) Cells adversely affected by penicillin, while unable to support the normal rate of phage multiplication are, nevertheless, lysed, perhaps even more rapidly than normally, certainly more abruptly.

(iv) The determination of growth curves for Staph. K phage in cultures of Staph. O, with and without the presence of penicillin in different concentrations and acting for different periods, have shown that the liberation of phage from those organisms initially infected occurs in bursts in all cases and after a constant latent period for given cultural conditions.

Penicillin at concentrations known to inhibit the continued growth of staphylococci does not significantly affect the rate and degree of multiplication of phage within the cells initially infected. Strictly, inhibition and eventual loss of viability may mean that the cell is so affected that it cannot divide more than once or twice and thus is rendered incapable of forming a visible colony. The fact that penicillin does not modify the first step in the phage growth curve is then not surprising. Penicillin in higher concentrations, however, does influence the yield of phage by each infected cell. After it has acted on the cell for periods comparable with the latent period of phage multiplication and mean generation time of the organisms, then the step-wise liberation appears to be replaced by a gradual process and finally there is no evidence of phage increase. Compared with the control, the yield of phage is very much reduced. Here, maybe, the $[P]$ is such that the synthesis of phage-precursor or essential substrate is rapidly retarded. This interpretation accords with the observations of Parker & Marsh (1946) on the lethal action of penicillin on staphylococci. In the (Staph. $O+P+\phi$) systems, cells not initially infected by ϕ are continuously under the influence of penicillin, whereas the corresponding cells in the control can multiply logarithmically. Hence the actual amount of 'lytic work' to be done by ϕ is greater in the absence of penicillin. The observed more rapid clearing of a Staph. O culture in $(\phi+P)$ systems than in systems where P or ϕ acts alone, is attributable, therefore, to the following factors:

(1) Penicillin inhibits the growth of the organisms initially not infected with phage so that at each stage lysis in the presence of penicillin produces a proportionally greater reduction in the turbidity.

(2) Cells whose growth has been affected through the action of penicillin, while they no longer support the normal multiplication of phage are, nevertheless, equally susceptible to lysis.

(3) The lytic potencies of penicillin and phage are supplementary.

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The Influence of Antibacterial Substances on the Interaction of Bacteria and Bacteriophages

2. Optical Studies of the Penicillin Effect

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SUMMARY: The ultra-violet light and electron microscopes were used to observe the effect of bacteriophage and penicillin on *Staphylococcus aureus.* Penicillin caused the organisms to swell to almost twice their normal size, and immediately before lysis they became less opaque to electrons and internal structure was evident. The triply segmented cocci seen in the ultra-violet light micrographs to be characteristic of penicillin-treated *Staph. aureus* appear to be due to the irregular arrest of the process of division, whereby a cell partly divides and then only one of the incipient daughter cells partly divides again.

The adsorption of the Staph. K phage to the surface of *Staph. aureus*, the latent period during which the coccus enlarges and the phage multiplies, and the lysis of the cell with liberation of phage particles were also recorded optically. Accelerated lysis of *Staph. aureus* by the combined action of phage and penicillin was not associated with any peculiarity in micrographic appearance, except that with high concentrations of penicillin the number of phage particles released on lysis was diminished.

During air- and freeze-drying the diameter of *Staph. aureus* contracts by 30–50%.

Electron micrographs of Staph. K bacteriophage, gold-shadowed and unshadowed show a round head totally opaque to 50 kV. electrons, 50–60 mμ. in diameter, and a slender tail 200–250 mμ. in length.

The various phases of the combined action of penicillin and bacteriophage on *Staphylococcus aureus* (Elford, 1948) have been examined by means of the ultra-violet light and of electron microscopes. These two instruments have their own particular merits and shortcomings for an investigation of this kind and it may be well to consider them briefly.

The ultra-violet light microscope. In this instrument, employing wave-lengths 275 and 253 mμ., the limit of resolution is decreased from 200 mμ. (that of the best visual microscope) to about 100 mμ. The specimen, dispersed in a medium transparent to ultra-violet light, is mounted between the quartz slide and cover glass, and examined either by directly transmitted ultra-violet light or the dark-ground method. The image presented by transmitted light is largely in terms of the specific absorption in the ultra-violet characterizing the various elementary structures in the object, whereas the dark-ground image is formed through the scattering of light. With biological specimens in particular, it is important to be able to study the object as nearly as possible in its natural state, i.e. in contact with its natural fluids or in a medium isotonic with them. Fixation treatment may alter structural details, and actual drying may completely transform the picture as the result of contraction, precipitation, crystallization and salting-out. As a means of minimizing these undesirable consequences the freeze-drying method suggests itself. Here it is hoped that by practically instantaneous freezing of the system and the subsequent removal of water by evaporation from the ice phase there will be little, if any, contraction and distortion of the main elements of structure. The ultra-violet light microscope provides a means of assessing the magnitude of the distortions due to such fixing and drying treatments; specimens of the same material can be examined in the untreated condition, and after drying in various ways, the object can be remounted in a suitable medium such as castor oil or glycerol water. This possibility holds, of course, only for objects of relatively large sizes within the resolution limits of the ultra-violet light microscope. Clearly, therefore, the effects on such objects as bacteria and tissue cells can be analysed.

The electron microscope. The resolution limit of this instrument in its present stage of development is probably about 1 mμ., thus providing a means of bridging the gap between the lower limit of application of the ultra-violet light microscope and the upper limits of X-ray analysis. However, from the viewpoint of biological studies the electron microscope has the serious drawback that the specimens must be in the dry state. Attempts have been made to investigate moist specimens enclosed between collodion film (Abrams & McBain, 1944), but the technical difficulties are great and it would appear doubtful whether the system would remain sufficiently stable for reliable examination. Some of the possible changes occurring on drying have already been indicated and it will be evident that in evaluating the results of electron microscopy these factors must be borne in mind. The treatment of the object before, and during, the actual mounting for electron microscope study should always be recorded in detail, otherwise there can be no reliable interpretation of the images photographed.

EXPERIMENTAL

Materials. The Oxford 'H' strain of *Staph. aureus* (abbreviation, Staph. O) and Staph. K bacteriophage (abbreviation ϕ) were used throughout, in papain broth, Hartley's broth and Fildes's defined medium. The penicillin was a pure sodium salt of penicillin G having an activity of 1600 international units (u.)/mg. For further details see Elford (1948).

Ultra-violet light microscope. The Beck-Barnard microscope (Barnard & Welch, 1936; Elford, 1938) was used with the condensed cadmium spark as the light source for the wave-length 275 mμ. The highest power Zeiss Quartz monochromatic lenses gave a resolution of 100 mμ. Photographs were taken at an initial magnification 1100.

Electron microscope. The R.C.A. model 'B' electron microscope was used, with no limiting aperture in the objective pole piece. The energy of the electron beam was 50 kV. throughout.

Specimen preparation. Specimens for examination in the electron microscope were mounted on thin films of collodion supported on a disk of fine nickel gauze (mesh 50 μ.). These films were prepared by allowing one drop of a 2 % solution of 'necoloidine' in amyl acetate to spread on the surface of water. Samples from the experimental systems were centrifuged and the deposit resuspended in water. This treatment was repeated and a drop of the resulting aqueous suspension then placed on the collodion film. It was either dried in air over P_2O_5 or freeze-dried. For the latter, the disk with specimen was placed on a copper block and left standing in a freezing-bath of CO_2-ethanol at $-72°$. The copper block with the frozen specimen was then transferred to a glass container in the vacuum line of a freeze-drying unit. The rate of evaporation was sufficient to keep the preparation frozen throughout the drying process, but the temperature was not kept at $-72°$. For study with the ultra-violet light microscope, specimens on quartz slides were similarly treated, and the dried organisms mounted in castor oil.

Experiments have been carried out by one of us (J. S.) to find out if it was possible to incorporate micro-organisms *in* a collodion film before dehydration by spreading 1 % collodion in amyl acetate directly on the moist specimen, and, if successful, whether distortion of the organisms and shrinkage in directions parallel to the plane of the film could be prevented. It was found that *Proteus vulgaris* and *Chromobacterium prodigiosum* could be mounted in this way, and that the resulting electron micrographs differed greatly from those obtained by the usual method of mounting described above. When air-dried on the surface of the film the organisms were totally opaque, or nearly so, to 50 kV. electrons, but when picked up from the surface of moist nutrient agar, or from a freshly prepared impression on glass, the organism yielded micrographs showing opaque internal structures in a relatively transparent cytoplasm. It was evident from the smooth outline of the organisms that serious distortion had been prevented and from the transparency of the cytoplasm that shrinkage accompanied by increase in mass density could not have taken place. Unfortunately, similar experiments with *Staph. aureus* were not successful.

RESULTS

Untreated staphylococci. Ultra-violet light micrographs of untreated Staph. O from cultures in the logarithmic phase of growth are shown in Pl. 1, fig. 1. The structure of the cell as it divides by binary fission is clearly seen. In contrast, the image of freeze-dried Staph. O in the electron microscope (Pl. 2, fig. 6), though indicating the shape and size, reveals nothing of the internal structure owing to the uniform opacity to 50 kV. electrons. A typical analysis of the size distribution in a normal Staph. O culture, from ultra-violet light micrographs, is given in Table 1, C; 80 % of the organisms have sizes in the range 0·75–0·90 μ. Measurements from electron micrographs of freeze-dried Staph. O yielded the corresponding figure 0·6–0·7 μ.

Staphylococci treated with penicillin. Penicillin in known concentration was allowed to act for a given period on Staph. O growing logarithmically in broth or synthetic medium and samples withdrawn for examination. In the presence of penicillin at 37° the staphylococci swell to almost twice their normal size and after prolonged contact are finally lysed. Typical analyses of size distribution are given in Table 1. Whereas in the early stages of penicillin action the electron microscope shows only the swelling of the organism (Pl. 2, fig. 7), the preparation being wholly opaque, the ultra-violet light microscope, on the other hand, does reveal internal structure (Pl. 1, figs. 2 and 3). The process of binary fission is seen to be anomalous when penicillin is present. Most of the enlarged cells appear to have developed to the point when fission should occur and then stopped, and there is often a noticeable difference in ultra-violet light absorption by the two halves of the cell. Also, many of the cells show three segments (Pl. 1, fig. 3), suggesting that growth has proceeded to the point of division, and one of the daughter cells, without being detached, has developed to the point of mitosis and no further. These observations support the view held by many workers that penicillin prevents normal division of the organism. Following prolonged action of penicillin the staphylococci become transparent to 50 kV. electrons and before disintegration internal structure can be seen with the electron microscope. Certain elements, presumably nuclear, retain their opacity and the cytoplasm contains fine granules which are liberated when the cell membrane disintegrates. Similar granules can also be discerned in the ultra-violet light micrographs (Pl. 1, fig. 3). These points are well illustrated in electron micrographs of material deposited by centrifugation from a culture lysed by penicillin (Pl. 3, fig. 8). These show cells in various stages of lysis, remnants of cells and yet other cells apparently highly resistant to the antibiotic.

Table 1. *Frequency distribution of the diameters of Staph. O treated in different ways, measured on ultra-violet light micrographs*

System	Diameter (μ.)										No. cocci measured
	0·30	0·45	0·60	0·75	0·90	1·05	1·20	1·35	1·50	1·65	
A. Untreated Staph. O, freeze-dried	16	25	54	4	1	—	—	—	—	—	256
B. Staph. O + penicillin*, 1 hr. at 37°, freeze-dried	—	1	23	52	24	—	—	—	—	—	250
C. Untreated Staph. O, papain broth culture	—	—	5	25	55	12	3	—	—	—	325
D. Staph. O + penicillin*, 1 hr. at 37°, papain broth culture	—	—	—	—	16	25	51	8	—	—	239
E. Staph. O + penicillin*, 3 hr. at 37°, papain broth culture	—	—	—	—	—	1	46	29	20	4	140

* 0·1 u./ml.

The influence of drying on staphylococci. Cultures of Staph. O in broth and defined medium were examined by ultra-violet light micrography, both as wet specimens, and remounted in castor oil after air- and freeze-drying (Pl. 1, figs. 1 and 4). The size distribution is given in Table 1, A and C. In an untreated culture of Staph. O 80% of the organisms have sizes within the ranges 0·75–0·9 μ., whereas the corresponding figure after freeze-drying treatment is 0·45–0·60 μ. Similar analyses of cultures containing 0·1 u./ml. penicillin (Pl. 1, figs. 2, 3 and 5; and Table 1, B and D) indicate that in the wet condition 75% fall within the size range 1·05–1·20 μ., while after freeze-drying the size range decreased to 0·6–0·75 μ. The contraction due to drying is therefore 30–50%.

Measurements on electron micrographs of freeze-dried organisms (*c.* 20 measured in each case) indicated the following peaks in the distribution of sizes. Untreated staphylococci, 0·6–0·7 μ.; staphylococci treated with 0·1 u./ml. penicillin for 1 hr. at 37°, 0·8–1·1 μ.; and after 3 hr. treatment with penicillin

1–1·25 μ. These figures are slightly greater than those determined in ultra-violet light micrographs. It may well be that the surface with which the organism is in contact during drying in some measure affects the final size. A small error in calibration may also be involved. In view of these facts the two methods may be said to yield results in fair agreement.

Staphylococci infected with bacteriophage. Young cultures of Staph. O at 37° were inoculated with concentrations of Staph. K phage sufficient to produce multiple infection. Electron micrographs of the phage adsorbed on to staphylococci are shown in Pl. 3, fig. 9. When infection has lasted for about half an hour the cell, enlarged relative to normal size, ruptures and an increased number of phage particles is released. No clear-cut evidence was obtained as to whether the phage multiplies at the surface of the cell or within the cytoplasm. In some instances the phage appeared to be congregated around the periphery of the cell, while in others the particles were located nearer to the 'nucleus'.

The elementary phage particles. Preparations of phage from filtrates of lysed cultures of Staph. O in synthetic medium, concentrated and washed with water in the air-driven centrifuge, were examined in the electron microscope. Staph. K phage comprises a round head opaque at 50 kV. electrons and a relatively long slender tail. Pl. 4, fig. 11, is a micrograph of a specimen treated with 0·02 M-$CaCl_2$ to enhance the contrast (Taylor, Sharp, Beard, Beard, Dingle & Feller, 1943). Measurements of the head indicated a diameter 50–60 mμ. and a tail length of 200–250 mμ. Pl. 4, fig. 12, shows Staph. K phage gold-shadowed by the method of Williams & Wyckoff (1945). The beaded appearance of the tail we consider to be an artefact due to the character of the metallic deposit. Pl. 4, fig. 10, is very interesting; a concentrated Staph. K phage preparation, washed several times in distilled water, has aggregated on the collodion with heads uniformly arranged as a layer over the film surface. The heads do not appear to be in contact, but are accompanied by an apparently fibrillar material. Further studies on concentrated preparations of this phage are in progress.

Staphylococci attacked by phage and penicillin. The general sequence of events following a combined attack by phage and penicillin on staphylococci differed little from that following infection by phage alone. After initial adsorption the majority of the particles was attached to the surfaces of the organisms and at the end of a definite latent period the enlarged cell disintegrated, liberating elementary phage particles. Lysis appeared to consist of a general dissolution of the cell wall and not the bursting of a bag that otherwise remained intact. After lysis, certain elements, possibly nuclear, that are opaque to electrons are frequently seen, similar to those remaining when penicillin or phage acts alone. The micrographs (Pl. 4, fig. 13) show cells soon after lysis by phage + 1 u./ml. penicillin. The escaping phage particles are clearly depicted, and appear to have been liberated in most cases from the peripheral regions of the cell, often as though propelled outwards as a wave but sometimes as a mass following the sudden dissolution of the cell. There is a wide variation in the number of particles escaping per cell, but the average figure for concentrations of penicillin not exceeding 0·1 u./ml. is much the same as for phage alone.

DISCUSSION

Our optical studies amply confirmed the conclusion drawn from direct analyses of the action, combined and singly, of phage and penicillin on staphylococci. Gardner (1940), Smith & Hay (1942) and Fisher (1946) have shown in microscopical studies that the organisms swell considerably in size under the action of penicillin. Weiss (1943) and recently Shanahan, Eisenstark & Tanner (1947) have used the electron microscope to study the effect of penicillin on various organisms. Their observation of the initial swelling of the organisms, culminating in the lysis of the cell, accords with our own. Our work with ultraviolet light micrography, in addition to confirming the general swelling of the organism in the presence of penicillin, throws further light on the stage at which growth and development of the organism are arrested; it is clear from the internal structure that the cell is unable to divide normally. It appears that one daughter cell, without being detached, is capable of further growth, again to the point of division. This leaves the enlarged cell divided into three segments, a picture characteristic of penicillin-treated staphylococci. Nothing similar was observed in any of our untreated staphylococci in the logarithmic growth phase. It would not be at all surprising, however, if such structures were occasionally encountered in untreated senescent cultures. In the period between the initial swelling of the cell in the presence of penicillin and lysis, the cell became increasingly transparent to 50 kV. electrons, so that an internal structure could be seen in electron micrographs, as, for example, in the disintegrating pair of cells (Pl. 3, fig. 8).

The electron microscope has been of great service in studying the process of infection of staphylococci by phage. It has shown the phage particles adsorbed to the cell surface, the enlarged cell during the latent period and, finally, elementary phage particles escaping from the cell on lysis (cf. Ruska, 1942; Luria, Delbrück & Anderson, 1943; Edwards & Wyckoff, 1947). The evidence is still not conclusive as to precisely where the phage multiplies. The balance of evidence favours the peripheral regions of the cell. If this be indeed the case, then the 'depressor effect' described by Delbrück (1945), whereby excess of superficially adsorbed phage influences the multiplication of intracellular phage, is more understandable. Unfortunately, the size of the phage particle is just below the resolution limit of the ultra-violet light microscope. Nevertheless, by photographing 0·1 μ. optical sections through the cell, the presence of adsorbed phage particles could be detected on the outer surface of the cell, though nothing definite could be learned regarding the whereabouts of particles within the cell.

The pictures of the combined action of phage and penicillin were very similar to those of phage acting alone, except that with high concentrations of penicillin the number of phage particles escaping from the lysed cell was less.

Staph. K phage was shown by means of the electron microscope to possess a round head, opaque to 50 kV. electrons, and a relatively long slender tail. The diameter of the head in the dried specimens is 50–60 mμ., a figure agreeing well with the size indicated by ultra-filtration and centrifugation data, 60 mμ.

and 70 mμ. respectively for the phage in its normal wet state (Elford, 1938). The presence of considerable excess of 'free tails' and the association of phage with masses of 'fibrillar' material, particularly in the early stages of purification, still leaves us in doubt as to whether the tail is an essential part of the phage's morphology. Further evidence is needed on this point.

The observations on the shrinkage and distortion of staphylococci during air- or freeze-drying are of importance in the interpretation of electron micrographs, particularly in estimations of size. The contraction of 30–50% on drying indicated in ultra-violet light micrographs is of the order of magnitude found by us for a number of micro-organisms studied by this method. Bacillary forms were particularly liable to distortion. Clearly, in any instance, the magnitude of the shrinkage and distortion will depend upon the object under investigation—viz. the rheological properties of the cell wall and of the cytoplasm, and on the density and size distribution of internal structure. Wyckoff (1946) has pointed out the need for minimizing the distortion due to desiccation, and has described a freeze-drying procedure very similar to our own. An additional advantage of the freeze-drying method in our experience was the more even distribution of organisms on the collodion film. It appears that any tendency to aggregation on the film, as may happen during the slower air-drying process, is largely eliminated by freeze-drying.

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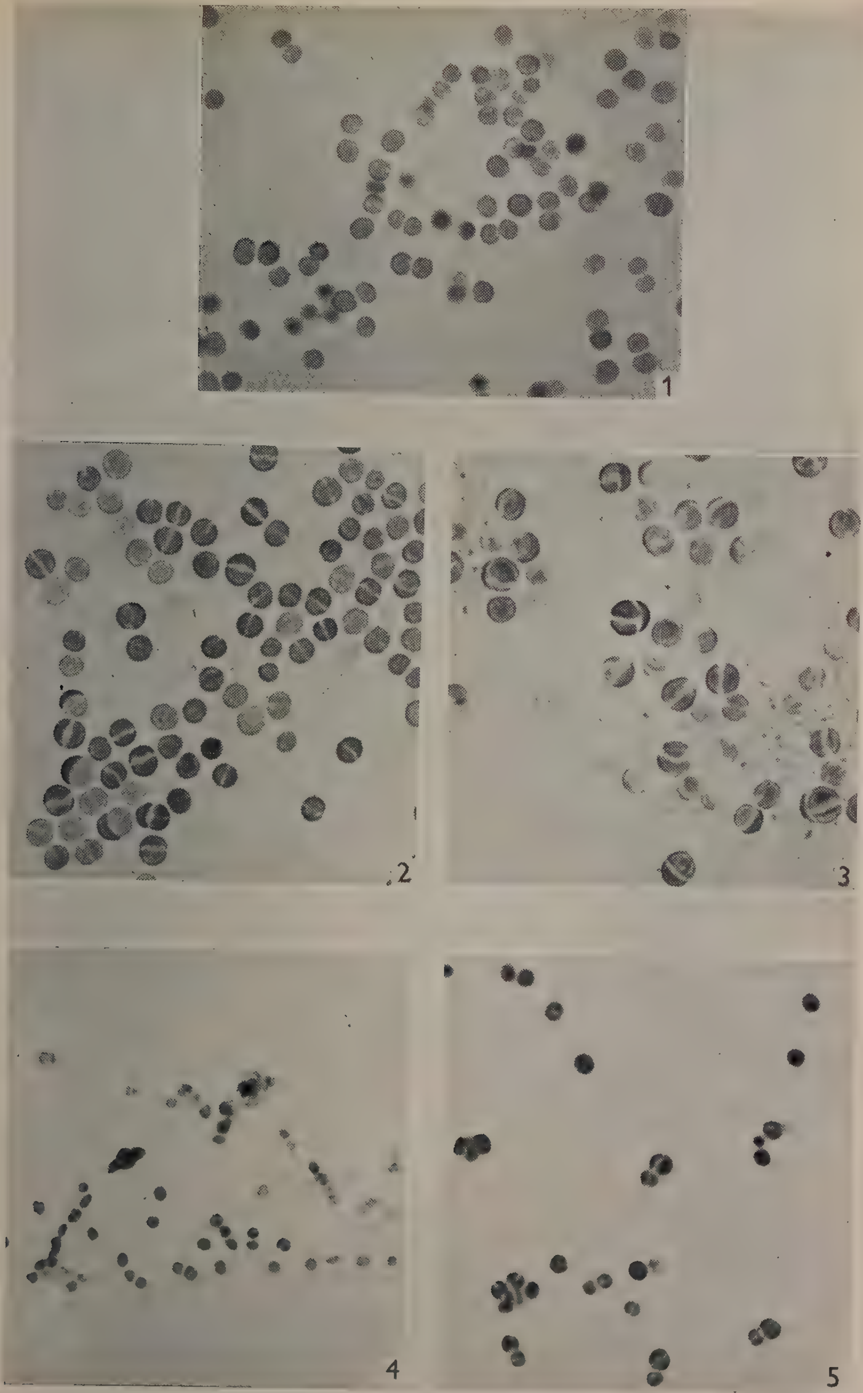
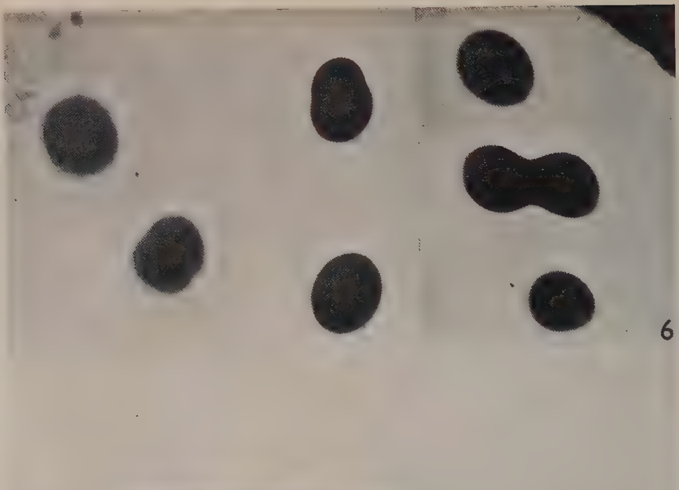


Figs. 1–5

J. Smiles, F. V. Welch and W. J. Elford—Penicillin and phage action. Plate 1



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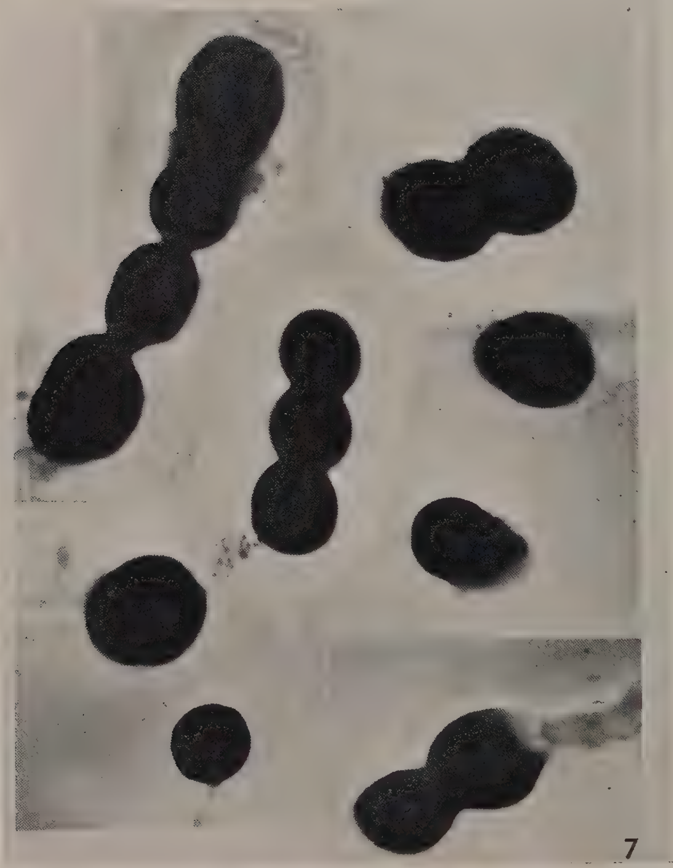

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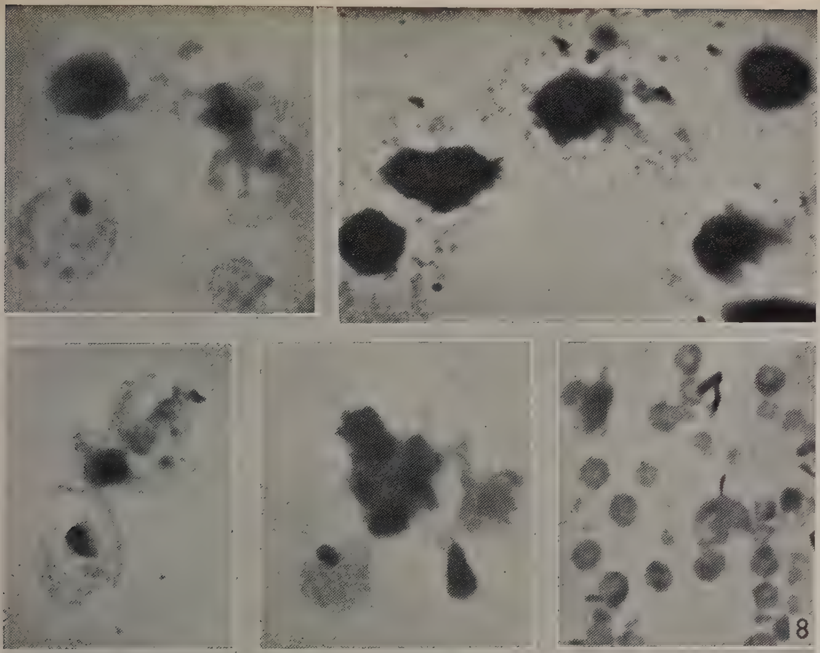
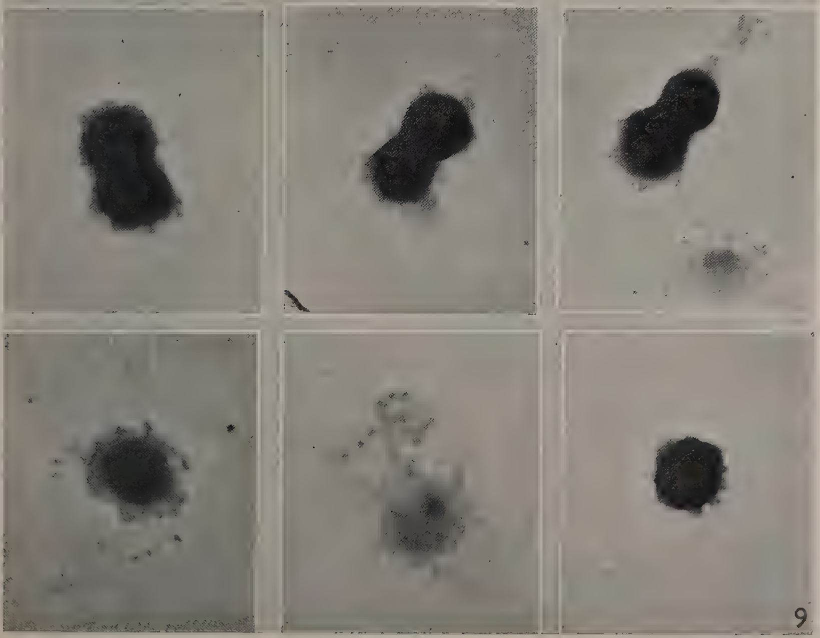
Figs. 6 and 7

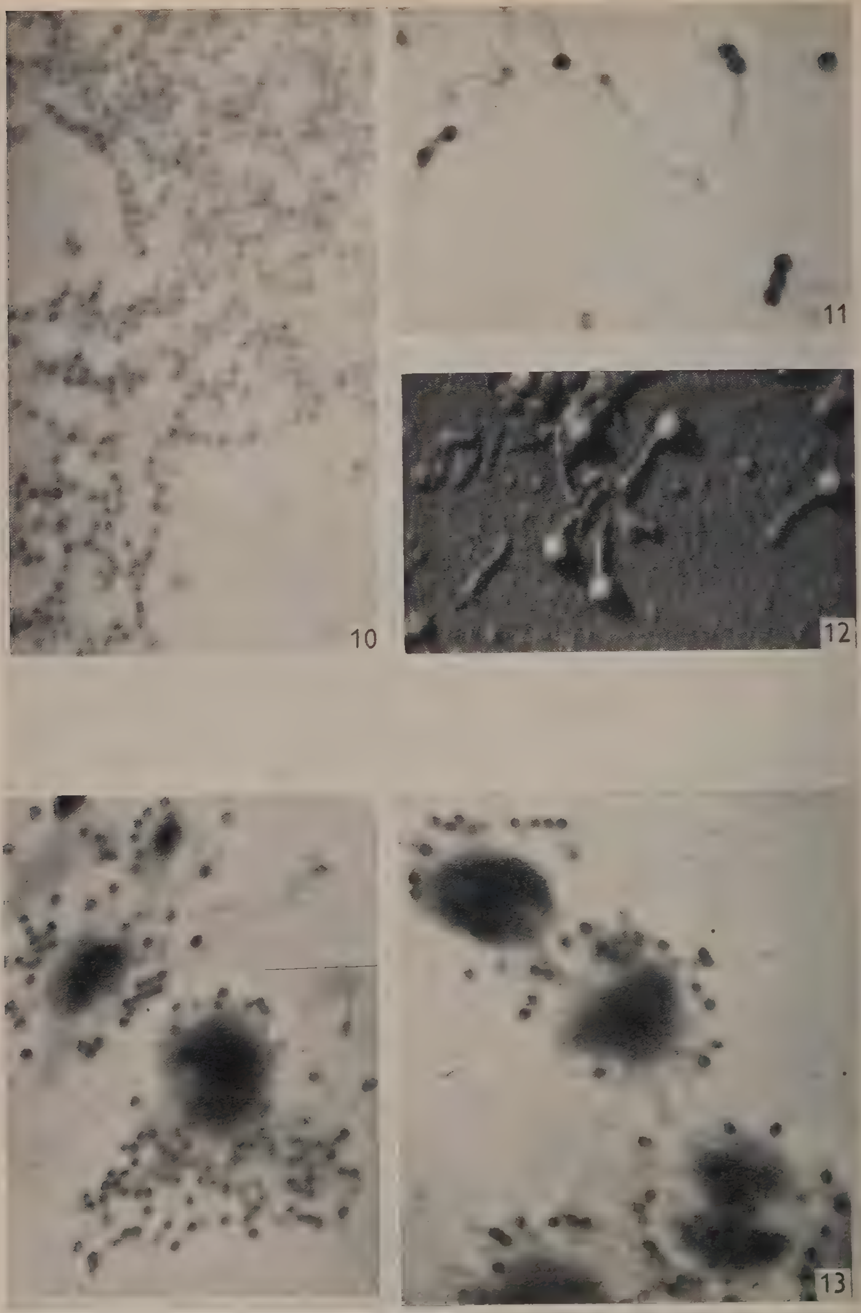
J. Smiles, F. V. Welch and W. J. Elford—Penicillin and phage action. Plate 2





Figs. 8 and 9

J. Smiles, F. V. Welch and W. J. Elford—Penicillin and phage action. Plate 3



Figs. 10–13

J. Smiles, F. V. Welch and W. J. Elford—Penicillin and phage action. Plate 4

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EXPLANATION OF PLATES

Plate 1

Ultra-violet light micrographs of *Staphylococcus aureus*, Oxford strain. Wave-length =275 mμ. Magnification × 3300.

Fig. 1. Staph. O 1 hr. culture in papain broth at 37°.

Fig. 2. Staph. O culture in papain broth incubated for 1 hr. at 37° in presence of 0·1 u./ml. penicillin.

Fig. 3. Staph. O culture in papain broth incubated for 3 hr. at 37° in presence of 0·1 u./ml. penicillin.

Fig. 4. Staph. O culture in papain broth for 1 hr. at 37°, concentrated by spinning and washed in water; freeze-dried and remounted in castor oil.

Fig. 5. Staph. O culture in papain broth incubated 1 hr. at 37° in presence of 0·1 u./ml. penicillin, concentrated and washed in water; freeze-dried and remounted in castor oil.

Plate 2

Electron micrographs of *Staphylococcus aureus*, Oxford strain. Magnification × 15,000.

Fig. 6. Staph. O from 1 hr. papain broth culture at 37°. Spun down and washed in distilled water; the film air-dried over P_2O_5.

Fig. 7. Staph. O from papain broth culture incubated 1 hr. in presence of 0·1 u./ml. penicillin at 37°. Spun down and washed in distilled water and the film freeze-dried.

Plate 3

Fig. 8. Electron micrographs of deposit remaining after the action of 0·1 u./ml. penicillin at 37° for 24 hr. on Staph. O. The lysed culture was centrifuged 15 min. at 10,000 r.p.m., the deposit washed twice in distilled water and the film freeze-dried. Magnification × 15,000.

Fig. 9. Electron micrographs of Staph. O and Staph. K phage (multiple infection, 10–20 phage particles/cell), showing phage adsorbed to surface of cell and the infected cell in different stages of disintegration. Magnification × 15,000.

Plate 4

Electron micrographs of Staph. K phage.

Fig. 10. Phage washed several times in distilled water in high-speed centrifuge. Air-dried on collodion film. Magnification × 30,000.

Fig. 11. Staph. K phage from lysed Staph. O in defined medium, centrifuged down and resuspended in 0·02 M-$CaCl_2$, again centrifuged and the deposit resuspended in water. Air-dried on collodion film. Magnification × 45,000.

Fig. 12. Staph. K phage from lysed Staph. O in defined medium, centrifuged and resuspended in water. Air-dried on collodion film and shadowed with gold. Magnification × 45,000.

Fig. 13. Staph. O attacked by phage and penicillin. Staph. O, 5×10^8/ml. in logarithmic growth phase in papain broth + Staph. K phage, 10^{10}/ml. + 1 u./ml. penicillin; incubated 45 min. at 37°, cells centrifuged and resuspended in water. Air-dried on collodion film.

Cells in various stages of lysis and phage particles being liberated. Magnification × 30,000.

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The Absence of Soluble Antibacterial Inhibitors in *Clostridium* spp.

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SUMMARY: By a cup-plate method, the antibiotic activity of fluid cultures of 253 strains of spore-bearing anaerobic bacilli representing over sixteen *Clostridium* spp. was tested against one strain each of *Mycobacterium phlei*, *Staphylococcus aureus*, and *Bacterium coli*. Two strains of *Clostridium sporogenes* and two of *Cl. bifermentans* showed a weak and variable activity against *Mycobacterium phlei*. The remainder of the tests were all negative.

In view of the current trends in antibiotic research it was thought that a survey of the antibiotic activity of spore-bearing anaerobic bacteria might yield something of interest. A large collection of *Clostridium* spp. isolated from war wounds was available for this purpose. A preliminary test of a few clostridial species in agar cup-plates, against *Mycobacterium phlei*, *M. smegmatis*, and *Bacterium coli*, revealed the presence of inhibitors of *M. phlei* in cultures of *Cl. sporogenes* and *Cl. bifermentans*. The methods of detection and titration of such inhibitors were developed using these strains. At first, the positive results with agar cup-plates were not obtained in repeated tests, although the plates were seeded with varying concentrations of test organisms, and three different media, Fildes's peptic digest (Fildes, 1921), a defined medium (Burkholder & McVeigh, 1942), and ox-heart extract nutrient agar were used. Titration in tubes, of gradocol membrane filtrates of broth cultures, however, revealed the presence of inhibitors, although in low concentration.

Detectable zones of inhibition on the agar plates were eventually obtained in nutrient ox-heart extract agar, seeded with approximately $0{\cdot}5 \times 10^6$ *M. phlei* cells/ml. The cups, 8 or 10 mm. in diameter, were cut with a cork-borer, and were filled with approximately 0·1–0·2 ml. of the culture to be tested. The plates were then held at 2° to enable any inhibitor to diffuse into the medium in adequate concentration before growth of the test organism commenced. The optimum period of chilling was proved to be about 2 hr. The plates were incubated aerobically, and it was not therefore necessary to remove the living anaerobic bacteria by filtration because they did not multiply appreciably during the incubation in air. The test cultures were grown in ox-heart extract cooked-meat broth, and after 2 and 6–7 days' incubation, samples were tested in three cup-plates containing, respectively, laboratory strains of *M. phlei*, *Staph. aureus* 'Oxford' strain, and *Bact. coli* 88. The *M. phlei* plates were read after 48 hr. incubation, the others after 24 hr.

RESULTS

The species, and the number of strains of each species examined were: *Cl. sporogenes* (60); *Cl. bifermentans* (46); *Cl. capitovale* (18); *Cl. welchii* (16); *Cl. multifermentans* (14); *Cl. cochlearium* (11); *Cl. hastiforme* (10); *Cl. histo-*

lyticum (8); *Cl. septicum* (8); *Cl. tertium* (6); *Cl. tetanomorphum* (5); *Cl. oedematiens* (3); *Cl. tetani* (2); *Cl. fallax* (2); *Cl. chauvoei* (1); and *Cl. butyricum* (1). In addition, forty-one strains, not identifiable as any of the above species, were tested; of these twenty-six were 'saccharolytic' and fifteen 'proteolytic'.

Four strains, two of *Cl. bifermentans* and two of *Cl. sporogenes*, were weakly active in plates against *M. phlei*, giving zones about 12 mm. overall diameter, corresponding to a titre of $\frac{1}{4}$ or $\frac{1}{8}$ in the tube tests. The production of inhibitors by these strains was, however, variable and did not necessarily occur on subsequent retesting. No inhibition of the staphylococcus or the coliform bacteria was observed.

The feeble activity displayed at times by these few strains did not warrant devising media to manifest the inhibitors with regularity or to produce them in workable quantity. It is possible that with other organisms and other conditions of test, more powerful inhibitors might have been found, but the results suggest that such substances are not generally produced by clostridia. Furthermore, various air-borne contaminants, cocci and bacilli, growing on the cup-plates in the early experiments, showed marked zones of growth stimulation extending into the zones of inhibition, suggesting that many bacteria yield antagonists to the inhibiting substances produced by *Cl. sporogenes* and *Cl. bifermentans*.

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The Breakdown of Phenols and Related Compounds by Bacteria

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SUMMARY: An *Achromobacter* strain isolated from soil grew on a mineral salt medium containing phenol or various phenol or benzoic acid derivatives as the sole source of carbon. The strain was moderately specific in its action on different classes of aromatic compounds. It did not break down unsubstituted aromatic hydrocarbons and their sulpho-, amino-, nitro- and halogen-derivatives, but did split phenols and carboxy-acids. This ability was limited to mono-cyclic compounds.

The microbicidal properties of phenol, which have been recognized for a long time, are in direct contrast to the fact that there are many types of bacteria which thrive in media containing phenols. It was observed that phenols added to the soil for the purpose of sterilization, instead of destroying the bacteria, actually encouraged growth (Russell & Hutchinson, 1909; Buddin, 1914; Mathews, 1924). The well-known fact that phenols arising in sewage or other decomposing matter fail to prevent the growth of bacteria further indicates the relative insensitivity of certain organisms towards these substances (Fowler, Ardern & Lockett, 1911).

Fuller investigation has shown that phenols are not only non-lethal to certain bacteria, but that they can even be decomposed by such organisms (Störmer, 1908; Wagner, 1914; Thornton, 1923; Grant & ZoBell, 1942). Gray & Thornton (1928), in particular, carried out an extensive research into this problem and isolated from soil various types of bacteria which were capable of using phenol or cresol as the sole source of carbon. These bacteria differed from one another in biological properties and in their ability to grow on different aromatic compounds. They readily tolerated concentrations of phenol up to 0·1 % and some could metabolize aromatic hydrocarbons also. In this connexion it must be mentioned that many authors have described bacterial strains which grow on aromatic hydrocarbons as their only supply of carbon, but these strains were unable to use oxygen-containing aromatic compounds (Söhngen, 1913; Taussen, 1929; Tausz & Donath, 1930; Frank & Goodale, 1942). It is obvious that soil bacteria exhibit a wide range of specificity towards different aromatic substances, but this problem awaits fuller investigation.

In the following experiments attempts have been made to investigate in one phenol-tolerant strain the relationship between chemical structure and the use of aromatic compounds by bacteria.

MATERIAL AND METHODS

Bacteria. Heavily manured garden soil was used as the source of bacteria. The specimens were mixed in 1 g. lots with 100 ml. 0·05 % phenol. After 3 days incubation at 24° one loopful was transferred to a mineral salt medium containing 0·05 % phenol as the sole source of carbon. In this way bacteria which used phenol were obtained.

The strains were plated on beef-extract peptone agar and single colonies subcultured in mineral salt medium containing 0·05 % phenol for the preparation of pure cultures. The adaptation of selected strains to higher concentrations of phenol was accomplished by subcultivation in mineral salt fluid-medium containing increasing amounts of phenol. The increase in the concentration of phenol was very small and did not exceed 0·005 % for each subculture. The subcultures were made every 4–6 days, owing to the very slow growth of these bacteria.

Only bacteria actually multiplying in 0·1 % phenol were used. The initial experiments were performed with five bacterial strains, all with the same biological features; for the more detailed experiments only the most actively growing strain was used.

The biological characteristics of the strains are as follows: Motile, Gram-negative bacilli, 2–4 μ by 0·5–1 μ, non-pigmented on ordinary nutrient agar; optimum temperature for growth about 22–24°; very scanty growth at 37°. After incubation for 24 hr. on agar the colonies are about 0·5 mm. in diameter, circular, smooth, greyish white and translucent. After 5 or 6 days the colonies are 3–5 mm. in diameter, raised, greyish, opaque, with smooth surfaces and regular edges. Uniform turbidity in broth with a granular deposit; on potato a greyish confluent growth; good growth on McConkey's medium, none on desoxycholate citrate agar medium. Milk slightly curdled, but no change in pH noticed. Gelatin liquified in 12 hr. Of the sugars examined (arabinose, rhamnose, xylose, glucose, mannose, lactose, maltose, sucrose, starch and inulin) only glucose was slightly fermented. No fermentation of alcohols (glycerol, adonitol, mannitol, dulcitol, sorbitol), or glucosides (salicin and aesculin), or of inositol. Indole not produced, and the Voges-Proskauer and methyl red tests negative.

On the basis of these biological characteristics the strains used were identified as belonging to the *Achromobacter* genus (Topley & Wilson, 1936).

Media. Two kinds of medium were used: (*a*) Nutrient (ox-heart extract peptone) agar; this was used for isolation of pure cultures, for standard cultures and for viability tests. (*b*) Liquid mineral salt medium based on a recipe of Gray & Thornton (1928) with the following composition: $MgSO_4$, 0·2 g.; $(NH_4)_2SO_4$, 1·0 g.; NaCl, 0·1 g.; KH_2PO_4, 1·76 g.; $CaCl_2$, 0·1 g.; Na_2HPO_4 (anhydrous), 2·88 g.; $FeCl_3$, 0·02 g.; distilled water 1000 ml.

In some experiments requiring unbuffered medium Gray & Thornton's original formula was followed.

Because precipitates form in the defined medium during autoclaving it was sterilized by filtration through Seitz-type pads. This medium, supplemented with 0·1 % of various aromatic compounds as the source of carbon, was used in the experiments on growth and adaptation to different aromatic compounds of the strains tested.

Inoculation. The synthetic media containing various aromatic hydrocarbons were inoculated with 0·01 ml. of a 14-day-old culture of the *Achromobacter* sp. grown on a medium containing 0·1 % phenol.

The measurement of bacterial growth. The growth of cultures was measured turbidimetrically, using a Spekker photoelectric absorptiometer, with smoky filters no. H. 508. Readings were made against distilled water. The intensity of turbidity expressed in units was read from a standard turbidity curve, which for comparison was calibrated against standard opacity tubes made according to Brown (1919).

In some experiments of long duration the turbidity alone was not an adequate measure because it did not exclude dead bacteria. To overcome this difficulty we counted the viable organisms by the plating method in addition, using an ordinary nutrient agar. The figures given on the viability curve (Fig. 1) were obtained by taking as an arbitrary unit the value recorded on the day before that on which a decrease was observed in the number of viable bacteria and expressing all figures obtained on subsequent days in terms of this unit.

Some of the aromatic compounds tested were only slightly water-soluble and produced opalescence in the medium, thus rendering turbidimetric estimations impossible. In these cases the bacterial growth was followed by direct counts.

Chemical estimations. A glass electrode was used for pH measurements.

Phenol was estimated by Theis & Benedict's colorimetric method (Theis & Benedict, 1924). All test compounds were purified and their purity was checked by melting- or boiling-point determination.

RESULTS

The initial experiments with the five different strains of *Achromobacter* sp. showed that all grew readily in 0·1 % phenol as the sole source of carbon; 0·15 % phenol prevented growth, but did not kill the bacteria until cultures were 10 days old; 0·2 % phenol was markedly bactericidal in 48 hr.

The same bacteria grew in the presence of glucose, acetate and citrate two to four times as abundantly as in the phenol medium. It seems that these aliphatic substances are a preferred source of carbon and that the use of phenol presents merely an alternative (emergency) mechanism of the bacterial cell, manifested distinctly only in the absence of easily metabolized aliphatic substances. When grown in medium containing 0·2 % of glucose and 0·1 % of phenol the strains used both substances simultaneously, but the breakdown of phenol was markedly decreased and the number of bacteria increased much faster than when phenol alone was the substrate. The multiplication of bacteria on phenol proceeded only when there was free access of oxygen.

The growth of bacteria on phenol as the sole source of carbon is slow during the first 2 days, passes during the next 7 days through the phase of logarithmic multiplication and reaches its peak in 10 days. The stationary phase lasts about 3 weeks, after which there is a slow decrease in the number of living bacteria (Fig. 1). The rate of growth on phenol was always reproducible if the same conditions were maintained.

The rate of disappearance of phenol in the medium is at first slow, but begins to be pronounced simultaneously with the phase of rapid multiplication of bacteria and increases progressively during the stationary phase as well (Fig. 1).

The *Achromobacter* strains, when growing on unbuffered mineral salt medium with an initial pH of 7·6, brought the pH down to 6·0 during the logarithmic phase and maintained it there during the stationary phase. The lowering of pH was much more pronounced in cultures growing on unbuffered media containing glucose instead of phenol. The use of phenol and the rates of bacterial multiplication were independent of pH within the limits of 6·0–7·6. Most of our experiments were performed in buffered media at pH 7·0.

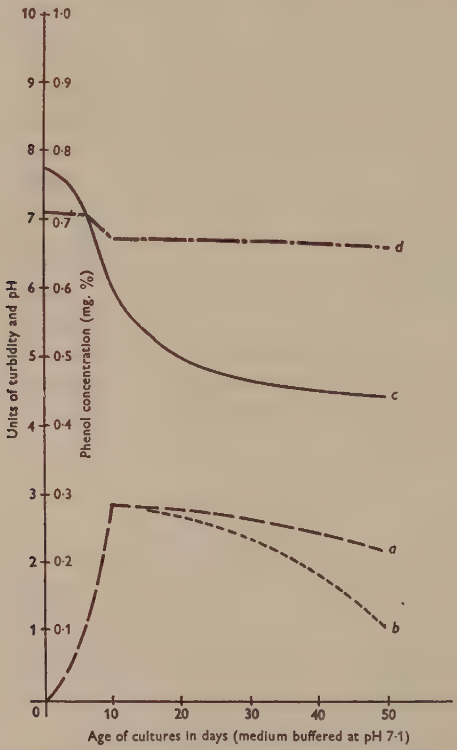

Fig. 1. *a* = bacterial growth estimated turbidimetrically; *b* = bacterial growth estimated by viable count; *c* = phenol concentration; *d* = pH of culture.

Table 1 shows the effect of various aromatic compounds as the sole source of carbon on one *Achromobacter* strain.

This strain is highly selective in using different aromatic compounds. It cannot use the unsubstituted aromatic compounds like benzene or toluene, either as free hydrocarbons or as their sulphonic acids, nor does the introduction into the benzene ring of chloro-, nitro, or amino-groups make the substances susceptible to breakdown by these bacteria. Hydrogenation of the benzene ring, however, does so, as was shown in the case of cyclohexane which is itself a good source of carbon. Nearly all the mono-, di- and tri-phenols investigated were good sources of carbon. It seems that the presence of free phenolic groups is essential. The phenolic ethers were not attacked by this strain, though the esters of phenol were readily used as a source of carbon.

Table 1. *Use of various benzene derivatives by an* Achromobacter *strain*

Compound	Bacterial growth after 5 days	Compound	Bacterial growth after 5 days
Benzene	—	Orcinol	+++
Toluene	—	Pyrogallol	+
Benzenesulphonic acid	—	Benzoic acid	+++++
p-Toluenesulphonic acid	—	Benzamide	+
Cyclohexane	+	*o*-Nitrobenzoic acid	±
Cyclohexanol	+	*o*-Hydroxybenzoic acid	++++
Monochlorobenzene	—	*p*-Hydroxybenzoic acid	±
Nitrobenzene	—	3, 5-Dihydroxybenzoic acid	+++++
Aminobenzene	—	*o*-Aminobenzoic acid	++
Phenol	+++	*p*-Aminobenzoic acid	±
Phenyl benzoate	++++	Phthalic acid	—
Phenyl acetate	++++	Terephthalic acid	—
Phenyl sulphate	+++	Phenylacetic acid	+++++
Phenylmethylether	—	Diphenylacetic acid	+++
Phenylethylether	—	Benzyl alcohol	+++
Diphenylether	—	Benzhydrol	±
o-Cresol	++++	Benzophenone	—
m-Cresol	++	Benzil	—
p-Cresol	++	*p*-Hydroxybenzaldehyde	±
3, 4-Xylen-1-ol	++	Naphthalene-2-sulphonic acid	—
3, 5-Xylen-1-ol	—	α-Naphthol	—
o-Dihydroxybenzene	+++	β-Naphthol	—
m-Dihydroxybenzene	+	1-Hydroxy-5-naphthoic acid	—
p-Dihydroxybenzene	—		

±, +, ++, +++, ++++, +++++, express the relative density of bacterial growth

Probably the enzymic system of the bacterial cell contains esterases which split the ester linkage, rendering the free phenol available to the bacterial cell, but there does not appear to be splitting of the ethers.

Quantitative estimations of bacterial growth showed that there were distinct differences in the rates of multiplication with different phenols. Although the limited accuracy of the method demands that conclusions should be drawn with caution, the results show that *o*-di-phenol (catechol) is used much better than *m*-di-phenol (resorcinol); *p*-di-phenol (hydroquinone) was not attacked by our *Achromobacter* strain. Fig. 2 shows the multiplication rate in different phenols.

Substitution of alkyl-groups in phenol had in some instances a marked effect on the availability of the compounds for our bacteria. The rate of growth with *p*- and *m*- methyl-phenol (cresol) is of the same order of magnitude as the rate of growth with phenol, but is lower than the rate of growth with *o*-methyl-phenol. The introduction of a methyl-group into resorcinol enhances its use by bacteria, orcinol being a much better source of carbon than resorcinol. There is no difference between the multiplication rate with 3, 4-xylen-1-ol and with phenol, but 3, 5-xylen-1-ol is not used at all.

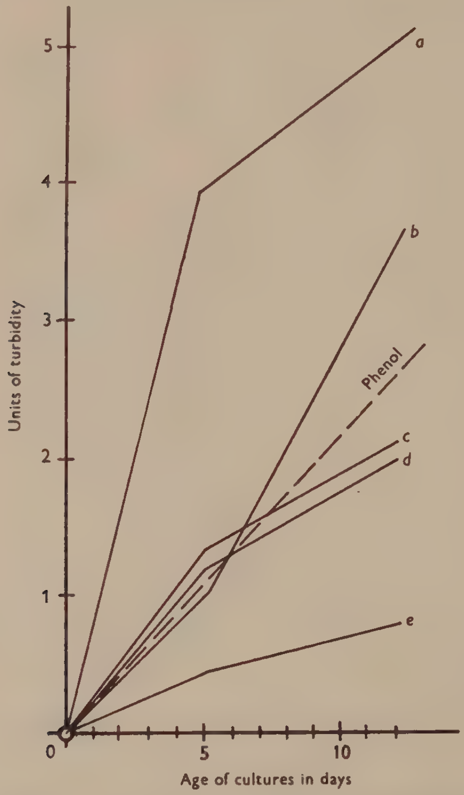

Fig. 2. a=o-cresol; b=m-cresol: c=catechol; d=p-cresol; e=resorcinol.

When a carboxyl-group is introduced into the benzene ring, the compound supports growth of our bacterium. The rate of multiplication on benzoic, phenylacetic and salicylic acids is distinctly higher than on phenol. However, in the case of carboxylic acid derivatives of benzene we must take account of many exceptions which make an explanation of the relationship between chemical structure and use by bacteria more difficult to formulate. For example the dicarboxylic acids, phthalic and terephthalic acids, were not used by our strain.

p-Aminobenzoic acid permits only very slight growth of the bacterium. This cannot depend upon the presence of an amino-group on the benzene ring, because the *o*-homologue of this acid is readily used. It seems, rather, that para-substitution in benzoic acid in general diminishes the availability of the compound for the bacterial cell (Fig. 3).

The alcoholic group also renders the benzene ring susceptible to the action of the bacterium, but this is not a general rule. Benzyl-alcohol is used, but not benzhydrol. It seems that the carbonyl-group does not alter the resistance of the benzene ring, since neither benzophenone nor benzil are used.

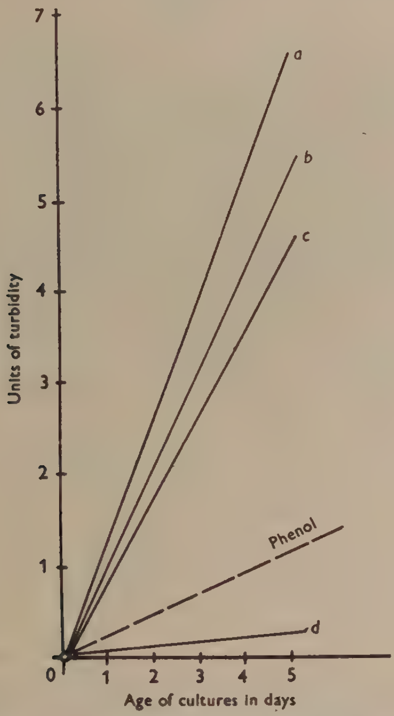

Fig. 3. a=phenylacetic and phenylbenzoic acids; b=phenyl benzoate; c=benzyl alcohol; d=p-aminobenzoic acid.

Only mono-cyclic aromatic compounds were susceptible to bacterial action; all the hydroxy- and carboxy-derivatives of naphthalene investigated failed to support bacterial growth.

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The Incidence and Character of Vibrios in British Waters

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SUMMARY: Forty-nine strains of vibrio were isolated from 32 of 43 samples of fresh water collected in various parts of England and Wales during the period October 1945 to July 1946. More than one kind of vibrio were found in 14 of the samples.

The types of vibrio isolated were heterogeneous in their fermentation reactions. Only three gave a cholera-red reaction. Twenty-five produced haemolysin for goat erythrocytes and all the haemolysins tested were filterable through gradacol membranes of 75 mμ pore diameter; an antiserum prepared against the haemolysin of one strain neutralized those of the other haemolytic water strains and of an El Tor vibrio.

Only one serological group of five strains related in 'O' and 'H' antigens was found; a few other strains showed H relationship and one (or two) possessed the H antigen of *Vibrio cholerae*; otherwise the organisms isolated were serologically heterogeneous with little antigenic similarity to one another or to known Asiatic vibrio types.

Compared with the extensive studies of the organisms of the *Vibrio* group which have been undertaken in India and the Far East, there is little information concerning their distribution and characters in western countries where cholera no longer occurs. In Britain, Houston (1913) examined a large number of London water samples, mainly from the Thames, for the presence of 'water microbes culturally resembling vibrios', but after finding 1885 strains atypical in the cholera-red test and in their behaviour towards gelatin he rejected them without further study.

The present investigation was therefore started with the following objects: (1) to determine the incidence of vibrios in open waters in this country; (2) to investigate their biochemical and serological characters; (3) to compare them with the true cholera vibrio and with water vibrios isolated in other parts of the world.

Method of isolation

Specimens were taken in ordinary unsterilized glass beakers, washed out several times in the water under examination. Each sample (180 ml.) was transferred to a sterile screw-capped bottle containing 20 ml. of an enrichment medium composed of 10 % peptone and 9 % NaCl in distilled water, with the pH adjusted to 9·2 and buffered with a boric acid solution, a few drops of thymol blue being added as indicator. The samples were usually collected during the afternoon and on returning to the laboratory the bottles were left on the bench at room temperature overnight. They were transferred to the 37° incubator at 10 a.m. the following day for 6 hr., after which a few drops of the surface scum was plated on Aronson agar. The bottles were again left on the bench overnight and a second plating was made the following morning.

Incidence of vibrios in water

During the period October 1945 to July 1946, 43 water samples from various parts of England and Wales were examined and vibrios were isolated from 32 of them. From 14 of the samples more than one type of vibrio was obtained (Table 1).

Although the media used were basically similar to those used in India by Taylor & Ahuja (1937), and found by them highly selective in their investigation of waters in Northern India, considerable difficulty was experienced in suppressing other water bacteria which tended to overgrow the more delicate vibrio. The procedure of isolation was adjusted from time to time. It was found that vibrios could often be picked up off plates, negative at first examination, which had been lying on the bench for several days, and it became the practice to examine all plates daily for a week before rejecting them as negative. This method was eventually adopted as giving the greatest number of positive results. It is probable that earlier failures to isolate vibrios from some of the samples were due to inadequacies of technique and that the incidence is actually higher than the 75 % found in this small survey.

At the beginning of the investigation, a period during which results were variable, the weather was mild and damp, but the samples from sources 22, 23 and 24, which failed to yield vibrios, were collected during a cold spell when there was snow on the ground, although no ice on the surface of the water. From March 1946 onwards all collections were made during warm weather and there were no vibrio-free samples. The series is, however, too small to allow any conclusions to be drawn about the influence of weather on incidence. Other observers (Houston, 1909; Taylor & Ahuja, 1937) were of the opinion that it did not play any definite part.

It is obvious, however, that organisms of the vibrio group are very widely distributed in the waters of this country and are to be found in such diverse sources as hill streams, rain-water collections and stagnant ponds.

Biochemical characters of the strains isolated

Fermentation reactions. The fermentation reactions of all the strains with mannose, sucrose and arabinose were studied and the vibrios classified according to the six types of Heiberg (1935). Organisms of type I formed by far the largest group, comprising 64 % of the whole series; 12 % only were of type II, and 16 % of type VI. There were none of types IV and V and only two organisms of type III. The non-fermenting group of type VI was subdivided into two subtypes according to the reactions with mannitol and glucose. Only one, no. 3*b*, fermented these sugars, thus falling into subtype 2, and the others remained non-fermenters of subtype 1. The overwhelming predominance of type I, to which *Vibrio cholerae* belongs, is of interest. Taylor, Read & Pandit (1936), in a study of the fermentation reactions of recently isolated vibrios from patients with cholera, carriers and water sources in the Calcutta area, found that only 11·4 % of vibrios isolated from waters belonged to type I, while 87 % were of type II. In a later study of water vibrios in Northern India, Taylor & Ahuja (1938) found that during the cold weather strains of type II formed only 6 % and type I 28 %. The incidence of type VI was also greater, forming another 28 % of the total, as against none in the Calcutta series and 13 % in Northern Indian waters examined during the hot weather and monsoon periods. In the present series six out of the eight type VI vibrio strains were isolated during the coldest part of the year, between the end of November and the beginning of February.

Table 1. *Waters investigated for the presence of vibrios*

No. of source	Source of water	Date of sampling	Vibrio strains isolated
1	Hampstead Heath duck pond (*a*), London	10 x. 45	1
2	Hampstead Heath swimming pond, London	10. x. 45	2
3	Hampstead Heath duck pond (*b*), London	10. x. 45	3*a*, 3*b*
4	Leg o' Mutton pond, Hampstead, London	16. x. 45	—
5	Stream, Golders Hill Park, London	16. x. 45	—
6	Pond, Barnet-bypass, Middlesex	17. x. 45	—
7	Pond, Mote Mount Golf Course, Barnet	17. x. 45	—
8	Serpentine lake, Hyde Park, London	23. x. 45	4*a*, 4*b*
9	Round Pond, Kensington Gardens, London	23. x. 45	5*a*, 5*b*
10	River Thames at Westminster Bridge, London	23. x. 45	6*a*, 6*b*
11	Fountain pool, Trafalgar Square, London	23. x. 45	—
12	Horse pond, Putney Heath, London	30. x. 45	—
13	Beverly Brook, Ranelagh Club	30. x. 45	7
14	Pond 1, Barn Elms Park	30. x. 45	—
15	Pond 2, Barn Elms Park	30. x. 45	8
16	Pond, Convent grounds, Roehampton	30. x. 45	—
17	River Thames at Hammersmith Bridge, London	30. x. 45	9
18	Canal, Regent's Park, London	5. xi. 45	10
19	Boating lake, Regent's Park, London	5. xi. 45	—
20	New River, Palmer's Green, London	20. xi. 45	11*a*, 11*b*
21	Mill stream, Bowcombe, S. Devon	9. xii. 45	12
22	Hill spring, Bowcombe, S. Devon	9. xii. 45	—
23	Cattle trough, Bowcombe, S. Devon	9. xii. 45	—
24	Spring, Bowcombe, S. Devon	9. xii. 45	—
25	Rain water from butt, Kingsbridge, Devon	21. i. 46	13*a*, 13*b*, 13*c*, 13*d*
26	Pond, Kingsbury, Middlesex	8. ii. 46	14*a*, 14*b*
27	Swift stream, slate quarry, N. Wales	24. iii. 46	15, 15*b*, 15*c*
28	Stream, Gate of Clwyd, N. Wales	24. iii. 46	22
29	Drinking trough, Llanbedr, N. Wales	24. iii. 46	16
30	North Lake, Kew Gardens, London	29. iii. 46	17
31	Stream, Rock Garden, Kew, London	29. iii. 46	18
32	South Lake, Kew Gardens, London	29. iii. 46	19*a*, 19*b*
33	Lily pond in hot-house, Kew, London	29. iii. 46	20*a*, 20*b*
34	River Thames at Kew, London	29. iii. 46	21*a*, 21*b*
35	Roadside stream, Totland, Isle of Wight	23. iv. 46	23
36	Ditch, Freshwater, Isle of Wight	23. iv. 46	24
37	Duck pond, Compton, Isle of Wight	23. iv. 46	25*a*, 25*b*
38	Horse pond, Yarmouth, Isle of Wight	23. iv. 46	26
39	River Thames at Tower Bridge, London	22. v. 46	27*a*, 27*b*
40	River Thames at Greenwich, London	22. v. 46	28
41	Stream, Worcester Park, Surrey	25. v. 46	29
42	River Thames at Richmond, London	11. vii. 46	30*a*, 30*b*
43	River Thames at Hampton Court, Surrey	11. vii. 46	31*a*, 31*b*

Cholera-red (*C.R.*) *and indole reactions.* The cholera-red reaction was carried out on 24 and 48 hr. cultures, in the nitrate peptone broth recommended by Bleisch (1898). In the whole series there were only three positive reactions, and all of these appeared at 24 hr. Two of the reacting strains were of Heiberg type I, and the other of type II. In the Indian series, during the hot weather and monsoon periods the incidence of positives was about 40 %, but in the cold-weather series no positive cholera-red reactors were found among the thirty-two strains examined.

Indole was produced by 32 % of the strains, including the three positive in the C.R. test.

Voges-Proskauer (*V.P.*) *test.* Barritt's (1936) modification was used as being more sensitive than the ordinary test, and the incidence of positives was 50 %. C.R. + strains also reacted positively in the V.P. test, and no Heiberg type I strain was C.R. + V.P. −, a biochemical combination which is considered by Taylor, Pandit & Read (1937) to provide a presumptive diagnosis of the serological O group I. On the other hand, the close parallelism found by these authors between the C.R. and V.P. tests has not been borne out in this series. Similar reactions were obtained with 27 of the vibrios and dissimilar reactions with 23 and, unlike the inagglutinable vibrios examined by them, a number of the C.R. − V.P. − group are of Heiberg type I.

Other biochemical tests. All strains were tested for their ability to liquefy gelatin, and to produce hydrogen sulphide. Only 22 % failed to liquefy gelatin, and 21 % of the strains were found to produce hydrogen sulphide after 3 days incubation at 37°.

The haemolysins of the strains isolated

All strains were tested for haemolysis at 2, 4, 24, 48 and 72 hr. The method was similar to that described by Goyle (1939), 1 ml. of a 5 % suspension of washed goat cells (packed centrifuged deposit v/v) being added to 1 ml. of peptone water culture. After 2 hr. at 37° the tubes were removed to the refrigerator, and the readings taken the following morning.

Approximately 50 % of the strains proved to be haemolytic, and of these all except two gave a positive reading at 24 hr. Greig (1914) and Goyle (1939), using Indian and Far Eastern strains, both found maximum haemolysin production in 72 hr. cultures, but Goyle also found that some of his weakly haemolytic strains had lost their haemolysin during the 3-day period of incubation, and therefore recommended that tests should be read at successive 24 hr. intervals. Taylor *et al.* (1937), in their study of the cholera-like vibrios, used a 48 hr. test as routine. The majority of the haemolytic strains in my series attained their maximum haemolysin production at 24–48 hr. and maintained it at 72 hr., and none which was haemolytic at 24 hr. had lost activity at 72 hr. On the other hand, one strain (30*a*) became haemolytic only at 48 hr., and two strains (9 and 10), though quite strongly haemolytic at 4 hr., were not haemolytic at 24 hr. Two of the strains, 3*b* and 6*a*, were found to be non-haemolytic when first isolated, but when retested several months later, haemolysed goat cells. This phenomenon has been observed by other authors.

The haemolysins of two of the strains, selected as being typical members of the series and strongly haemolytic, were further investigated and found to fulfil the criteria required of a true toxin in that they were heat labile, being destroyed by heating at 56° for 10 min., filterable, and antigenic.

Filterability of the haemolysin. Greig (1914), in his original experiments, filtered haemolytic vibrio cultures through Pasteur-Chamberland candles and, testing the filtrates, found that a considerable diminution had occurred in the process, or in a few cases complete loss of haemolytic activity. He concluded that 'the haemolysin producing substance...is to a considerable extent non-filterable'. Goyle (1939) used a Pasteur-Chamberland candle, a Seitz E.K. disk and a Berkfeld N filter, and collected the filtrates in successive 10 ml. fractions. He found that the haemolysin was definitely filterable, but tended to be adsorbed on the filter disks or candles. This adsorption was most marked with the Seitz disks and least with the Berkfeld N filter, though even when the latter was used the final filtrate fractions did not attain the haemolytic titres of the unfiltered cultures. In the present investigation supernatant fluid from a centrifuged haemolytic peptone water culture was passed through an Elford gradacol filter membrane of $0{\cdot}75\,\mu$ pore diameter. The filtrate was collected in successive 10 ml. fractions and tested for haemolysin in parallel with the unfiltered culture. The first fraction was found to be only weakly haemolytic, with a titre of only 1/1 as against 1/512 of the unfiltered culture, the second produced haemolysis at a dilution of 1/32, and the third was as powerfully haemolytic as the parent culture. The superiority of this type of filter for the purpose was thus demonstrated and Goyle's claim of the filterability of the haemolysin was confirmed.

Antigenicity of the haemolysin. Using Goyle's method, an anti-haemolytic serum was prepared against vibrio 6*b* and tested against the haemolysins of other vibrios in the series. The anti-haemolytic activity of the serum was not as marked as in Goyle's sera, and a serum concentration of 1/10 was required for complete inhibition of the haemolysin of the vibrio 6*b*. Control series were therefore run with all tests, using 1/10 normal serum from the same rabbit. It was found that the serum inhibited the haemolysins of all the British water vibrios and also that of the only El Tor vibrio tested (strain Doorenbos 34D13).

Serological characters of the strains isolated

Antisera were prepared against 23 of the British strains (19 against the O antigens and 11 against the whole organisms), against the typical cholera vibrios 'Inaba' and Ogawa 1077 (O and OH antisera) and against the O antigens of nine strains representing as many of Taylor & Ahuja's (1937) Indian vibrio types from water, from carriers, and patients with cholera.

The agglutination technique was similar to that described by Taylor *et al.* (1937). Suspensions of agar-grown vibrios in 0·25 % formol saline were made of all the British strains, of the true cholera vibrios and of 22 'inagglutinable' Indian strains from various sources.

After preliminary selection by slide agglutination tests, tube agglutination

Table 2. *Biochemical and serological characters of British water vibrios*

	Biochemical reactions							Agglutination by			
								O antibodies of		H antibodies of	
Strain no.	Heiberg type	Cholera-red reaction	Voges-Proskauer test	Liquefaction of gelatin	Production of H_2S	Haemolysin	Indole formation	Other British vibrio strains	Asiatic vibrio* strains including *V. cholerae*	Other British vibrio strains	*V. cholerae* (%)
1	I	—	+	+	—	+	—	2, 7, 9, 10; 100 %	—	2, 7, 9, 10; 100 %†	—
2	I	—	+	+	—	+	—	1, 7, 9, 10; 100 %	—	10; 100 % 1, 7, 9; 100 %+	<25
3*a*	I	—	—	+	+	+	+	26, 21*b*; <25 %	—	—	—
3*b*	VI	—	—	+	—	+‡ (−)	+	—	—	—	—
4*a*	I	—	+	+	—	+	—	—	—	—	—
4*b*	I	—	+	+	—	+	—	—	—	6*b*; 40 %	—
5*a*	I	+	+	+	—	+‡ (−)	+	—	—	5*b*; 50 %	100
5*b*	I	—	+	+	+	+	—	24, 1, 2; <25 %	V. tank 4B; 25 %	5*a*	50
6*a*	II	+	+	+	—	+	+	—	V. MW/166; <25 %	10‡; <25 %	<25
6*b*	II	—	+	+	+	+	—	—	—	4*b*; 40 % 15*a*, 15*b*; 100 %† 12; <25 %	<25
7	I	—	+	+	—	+	—	1, 2, 9, 10	—	1, 2, 9, 10	—
8	III	—	—	+	—	—	+	—	—	10; 40 %	—
9	I	—	+	+	—	+	—	1, 2, 7, 10	—	1, 2, 7, 10	—
10	I	—	+	+	—	+	—	1, 2, 7, 9	—	2; 100 % 1, 7, 9; 100 %†	<25
11*a*	VI	—	—	—	+	—	—	—	—	—	—
11*b*	III	—	+	+	—	—	—	—	—	—	—
12	I	—	—	—	—	—	—	—	—	6*b*; <25 % 2, 10; 40 %	—
13*a*	I	—	+	+	—	+	—	—	V. tank, 4B V. 1612 } <25 %	—	—

13*b*	VI	−	−	−	+	−	−	—	—	—	—
13*c*	VI	−	−	−	−	−	−	—	—	—	—
13*d*	VI	−	−	−	+	−	−	—	—	—	—
14*a*	VI	−	−	−	+	−	−	25*a*; 40 %	—	—	—
14*b*	VI	−	−	−	+	−	−	—	—	—	—
15*a*	I	−	+	+	−	+		—	—	6*b*; 100 %†	—
15*b*	I	−	+	+	−	+		—	—	6*b*; 100 %†	—
15*c*	I	−	−	+	+	−		—	—	—	—
16	I	−	−	+	+	−		—	—	—	—
17	II	−	+	+	+	−		—	—	—	—
18	I	−	−	+	+	−	+	—	—	—	—
19*a*	I	−	+	+	+	−	+	—	—	—	—
19*b*	I	−	−	+	+	−	−	—	—	—	—
20*a*	II	−	−	+	+	−	−	18*a*; <25 %	V. 630/3T; <25 %	11*b*; < 25 %	—
20*b*	II	−	−	+	+	−	−	—	—	—	—
21*a*	I	−	+	+	+	+	−	—	—	—	—
21*b*	I	−	+	+	+	−	+	3*a*; <25 %	—	—	—
22	I	+	+	−	+	−	+	—	—	—	—
23	I	−	−	−	+	−	+	—	V, 630/3T; <25 %	11*b*; <25 %	—
24	I	−	−	+	−	−	+	5*b*; <25 %	—	—	—
25*a*	I	−	+	+	−	+	−	14*a*; 40 %	V. Nanking 32/124; <25 %	—	<25
25*b*	I	−	+	+	−	−	−	—	—	—	—
26	I	−	−	+	−	−	+	3*a*; <25 %	—	—	—
27*a*	I	−	−	+	−	+	−	—	—	—	<25
27*b*	I	−	−	+	−	+	−	—	—	8; <25 %	—
28	I	−	−	−	−	+	+	—	—	—	—
29	VI	−	−	+	−	−	−	—	—	—	<25
30*a*	II	−	−	+	−	+	−	—	—	—	—
30*b*	I	−	+	+	−	+	+	—	—	10; <25 % •	—
31*a*	I	−	−	+	+	+	+	—	—	—	—
31*b*	I	−	+	+	−	+	+	3*a*; <25 %	—	10; <25 %	—

* The thirty or more vibro types (other than *V. cholerae*) serologically differentiated by Taylor and his co-workers in India were numbered variously from 1 upwards in the original communications and to avoid confusion are referred to here by the name or number of the original strain.

† Absorption of agglutinins by heterologous suspension incomplete.

‡ Negative when first isolated, but positive when re-examined several months later.

<25% Indicates between 15 and 25 %.

tests were made with all positive reactors, using serum dilutions between 1/25 and 1/25,000. The tubes were incubated in a water-bath at 52° for 4 hr. and readings taken at this term and again after the tubes had stood on the bench overnight.

The results of these agglutination tests are summarized in Table 2. For simplicity cross-reactions amounting to more than 15 % but less than 25 % of the homologous titres of the sera concerned are recorded by the sign $<25\,\%$, while those falling below 15 % are entered as negative. It is thought that cross-reactions of this order are not sufficiently significant as indices of relationship to warrant their more detailed statement.

As indicated in the table, the British water vibrios proved to be serologically heterogeneous. Of the 50 strains only five (1, 2, 7, 9 and 10) were closely related in both their O and H antigens, forming a compact group. Two others (14*a* and 25*a*) showed a measure of similarity limited to the O antigens; another three or four (4*a*, 6*a*, 15*a* and 15*b*) showed relationship restricted to the H antigens, and the same was true of the strains 5*a* and 5*b* in which the H antigen appeared to be identical with, or to resemble closely, that of *V. cholerae*. The remaining races were apparently strain specific.

None of the strains reacted appreciably with cholera O agglutinins and only two to as much as 25 % of the homologous titre with any of the O antisera prepared against Indian and Far Eastern vibrio types—vibrio 23 with the O antiserum of vibrio 630/3T (a Calcutta 'case' strain, 'strain 9' of Taylor *et al.*), and vibrio 25*a* with that of vibrio Nanking 32/124 (also a 'case' strain, Group II, Gardner & Venkatraman (1935), and 'strain 6' of Taylor *et al.*).

Phage sensitivity of the strains

All the strains were tested, as nascent agar-plate cultures, with droplets of concentrated preparations of the Indian cholera phage types A–L and three were found sensitive to certain of these.

Strain 5*a*	reacted with	phage type	B	to give	'cloudy lysis'
Strain 6*a*	,,	,,	B	,,	total clearing
	,,	,,	F	,,	partial clearing
	,,	,,	J	,,	partial clearing
Strain 22	,,	,,	B	,,	partial clearing
	,,	,,	J	,,	partial clearing

It is interesting to note that these three strains, 5*a*, 6*a* and 22, were the only members of the series to give a positive cholera-red reaction.

I wish to thank Major-General Sir John Taylor and Mr P. Bruce White for their invaluable encouragement and advice, and Miss Irene Gammon, Miss Edna Schultze and Miss Marie French for their technical assistance.

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Nuclear Fusion and Reorganization in a Lactobacillus and a Streptococcus

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SUMMARY: There is an apparent cycle of nuclear reorganization in *Lactobacillus* sp. Nuclear fusion occurs between the units of a multicellular bacillus. The organism then increases in length, the nucleoplasm being redistributed throughout the resulting filament, which by fragmentation returns to the bacillary condition. A very similar cycle is seen in *Streptococcus faecalis*.

Although the subject has not been very fully studied, a number of observations suggestive of nuclear reorganization in bacteria have been made. Stoughton (1932) and Braun & Elrod (1946) produced strong evidence of conjugation in phytopathogens. Badian (1933) and Klieneberger-Nobel (1945) described a process of nuclear fusion as a preliminary to sporulation in species of *Bacillus* and *Clostridium*. Although sporulation was described as autogamous, it has been observed that bacilli of the types studied by Badian and Klieneberger-Nobel are usually multicellular (Robinow, 1945; Bisset, 1947), so that it is not unlikely that conjugation between the cells of multicellular bacilli was in fact observed.

The regular, if infrequent, occurrence in bacterial cultures of forms suggestive of such nuclear changes, led the author to search for a non-sporing species in which their occurrence was sufficiently frequent to enable them to be studied with advantage. These requirements were fulfilled by a number of strains of *Lactobacillus* sp., isolated in almost pure culture from a high proportion of samples from carious teeth. Observations were made upon 10 different strains which, although by no means identical, were all small, Gram-positive, acidophilic bacilli, of the general appearance of lactobacilli. In view of our limited knowledge of the taxonomy of this group, further identification was not attempted. Observations were also made upon a strain of *Streptococcus faecalis* in which similar forms were found.

METHODS

The bacteria were cultivated aerobically at 37° upon nutrient agar containing 0·2 % glucose, at pH 5. Growth in 24 hr. under these conditions was slight; no growth at all was obtained at pH 7. Cultures were examined at various ages from 4 to 72 hr., but those from 18 to 24 hr. old were found to be most productive of the types of cell which it was desired to examine.

Films were stained by Robinow's (1945) modification of the Feulgen technique. Robinow's tannic-acid-violet technique for the demonstration of cell walls was also employed. Wet preparations were always used.

OBSERVATIONS

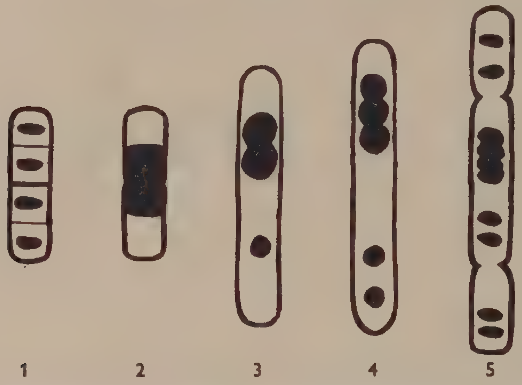
The lactobacilli were of Rough morphology, they formed 'medusa-head' colonies and possessed the multicellular structure and transverse septa typical of this state (Bisset, 1938, 1947) (Pl. 1, fig. 1). The chromatinic bodies were also arranged in the usual pattern of a Rough bacillus, each of the two or four cells of a bacillus containing a single body, probably an unresolved pair of chromosomes.

The bacteria in which apparent nuclear reorganization was observed were of two main types: (1) bacilli in which all the nuclear material appeared to have fused in the central region (Pl. 1, figs. 2–11); (2) filamentous organisms (Pl. 1, figs. 12–16). In the first type the size and appearance of the fused mass was variable. Usually there was some degree of central constriction, and sometimes the separate bodies composing it could be distinguished (Pl. 1, figs. 3, 5, 10). Most frequently the dimensions were those of a four-celled bacillus, but some were little bigger than a two-celled bacillus. Comparison can readily be made in Pl. 1, fig. 2, where one of these forms and a typical four-celled bacillus are lying side by side. It should be noted that the apparent gap between the two halves of the latter represents a transverse septum, continuous with the cell wall (Pl. 1, fig. 1). The membranous septa which subdivide the two halves are better shown in the dividing bacillus on the right of Pl. 1, fig. 3. In the second type the organisms varied in length from a little longer than the preceding type, to filaments, apparently unicellular, and as much as six or seven times as long. The smaller filaments contained one or more large, discrete masses of chromatinic material, and occasionally smaller, scattered masses (Pl. 1, figs. 12–15). In some of the larger filaments the chromatinic material was more evenly distributed, and signs of fragmentation of the filament were observed (Pl. 1, fig. 16).

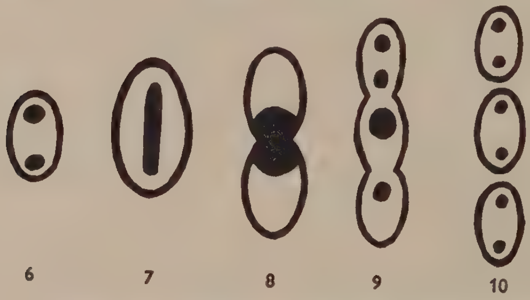
Similar observations were made upon a strain of *Strep. faecalis* (Pl. 1, figs. 17–22). In this case the original fusion nucleus appeared to consist of a longitudinal bar of chromatinic material (Pl. 1, figs. 17, 18). The other forms which were observed closely resembled those seen in the lacto-bacilli, except that the lanceolate form of the coccus was always discernible, and the filaments formed were seldom very long (Pl. 1, figs. 19–22).

DISCUSSION

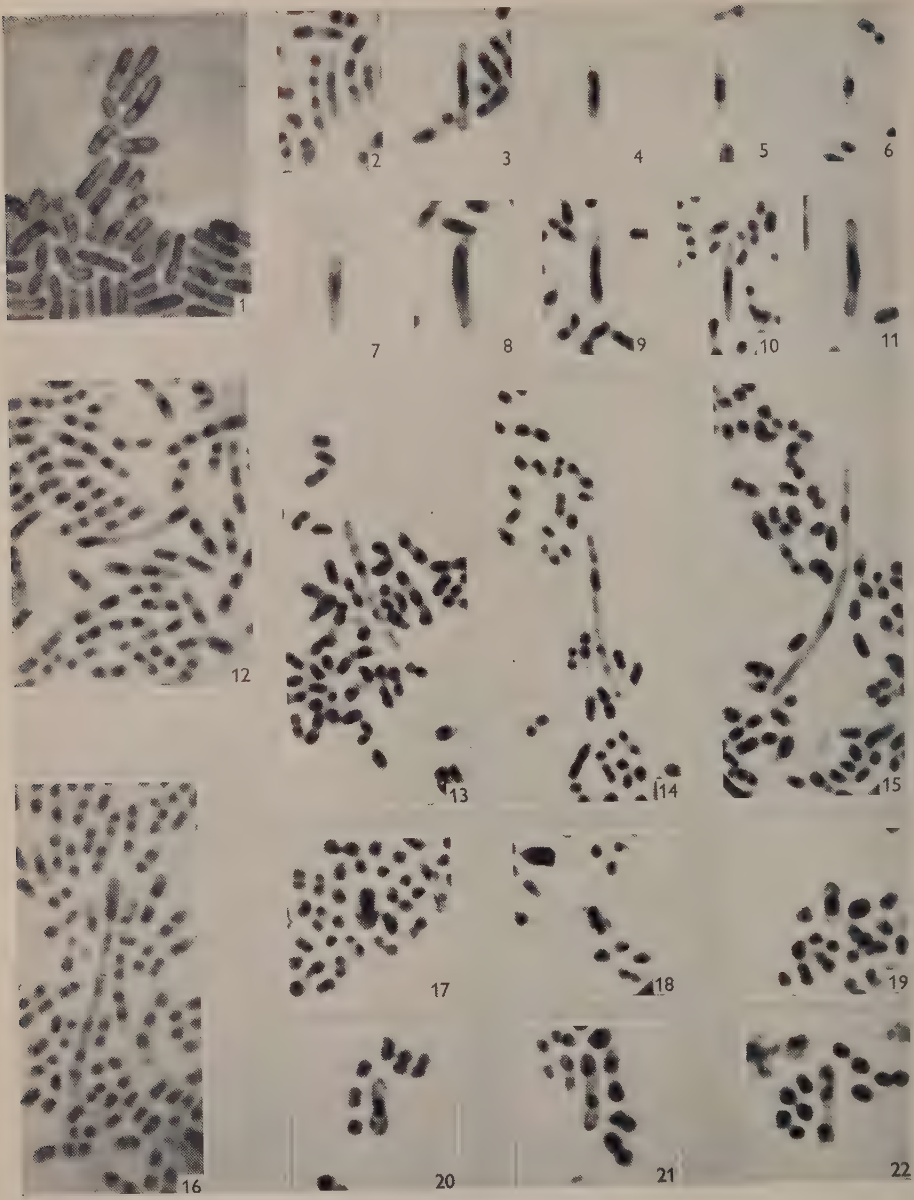
It appears logical to assume that a long filament is most probably derived from a shorter one, and can become short only by fragmentation, or by rejection of a portion of its material. Shrinkage in length alone is unlikely. It is therefore probable that the bacilli which contain the central mass of chromatinic material are derived from a normal bacillus (Figs. 1, 2), and grow into short filaments in which the chromatinic material is partly redistributed (Figs. 3, 4). These are transformed into longer filaments, where redistribution is completed and which return, by fragmentation, to the original bacillary condition (Fig. 5). The process in the streptococcus (Figs. 6–10) appears to follow a very similar pattern, although it is not clear whether the fusion nucleus is derived from a single coccus or, by conjugation, from more than one. A similar process was tentatively suggested by Robinow (1944) in his descriptions of certain types of cell found in cultures of *Escherichia coli* and *Proteus vulgaris*. His figures appear to represent similar forms to those described in the present paper, and he refers to the final step, the fragmentation of the filaments, in another publication (1947). The process also bears a resemblance to that described by Klieneberger-Nobel (1945) as a preliminary to sporulation, at least so far as the original nuclear fusion and redistribution is concerned. The reduction of chromatinic material which accompanies the actual process of sporulation has no obvious parallel in the present case, although it is not impossible that it may occur in the process of fragmentation, or by absorbtion of nuclear material in the cytoplasm in the manner described for *Cytophaga* (Krzemieniewska, 1930). This possibility is perhaps enhanced by the fact that the nuclear reorganization of *Cytophaga* takes place in a filamentous type of cell. It is also conceivable that the nuclear material in the filamentous form is not exclusively derived from the redistribution of the fusion nucleus, but that some portion arises



Figs. 1–5. Suggested interpretation of nuclear reorganization in *Lactobacillus* sp. (diagrammatic).



Figs. 6–10. Suggested interpretation of nuclear reorganization in *Strep. faecalis* (diagrammatic).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Figs. 1–22

K. A. BISSET—NUCLEAR FUSION AND REORGANIZATION IN A LACTOBACILLUS. PLATE 1 *de novo* in the cytoplasm, or from structures analogous to a nucleolus. Studies by the author of these processes as they occur in Gram-negative, intestinal bacteria (Bisset, 1948) suggest that the chromosomes, which behave throughout as paired structures, divide once in the fusion nucleus and once during the redistribution; thus each chromosome of the three pairs which take part in the formation of the fusion nucleus gives rise eventually to a single bacillus containing two pairs of chromosomes. In the case of the *Lactobacillus* sp., where the bacillus contains four cells, each with a single pair of chromosomes, the details must be rather different, although, where they can be resolved the chromosomes appear to follow a similar general plan.

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EXPLANATION OF PLATE

All × 3000

Fig. 1. *Lactobacillus* sp., preparation of cell walls, showing septa. Tannic-acid-violet.

Figs. 2–11. *Lactobacillus* sp., forms showing fusion nuclei. Acid-Giemsa.

Figs. 12–16. *Lactobacillus* sp., filamentous forms showing redistribution of nuclear material. Acid-Giemsa.

Figs. 17–18. *Streptococcus faecalis*, forms showing fusion nuclei. Acid-Giemsa.

Figs. 19–22. *Strep. faecalis*, filamentous forms showing redistribution of nuclear material and fragmentation of filaments. Acid-Giemsa.

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Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

3. Variation in the *Actinomyces coelicolor* species-group

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SUMMARY: Variation in the *Actinomyces coelicolor* species-group comprises loss of pigment and aerial mycelium, and occasionally of agar liquefaction. Stable variants may arise from degenerate, aged, vegetative mycelium, but do not normally do so when the vegetative mycelium is kept in vigorous condition by frequent subcultivation in suitable media. Single spore isolations from the aerial mycelium of typical and of variant colonies show that there may be inherent differences in the sister-spores of the same chain. Thus, in an agar-liquefying strain 3 of 15 spores had lost the power to produce pigment and to liquefy agar; and an atypical colony of the same strain yielded three viable isolates each with a strong tendency towards sectoring, with the ultimate production of a colourless, non-agar-liquefying variant as well as the typical growth. A non-agar-liquefying strain, which by prolonged cultivation in the vegetative phase had lost its power of producing the red-blue indicator pigment, yielded a variant giving rise to sectored colonies with occasional restoration of the blue pigment. Spontaneous occurrence of variants may be detected in certain spores of the aerial mycelium of a well-grown typical colony, although it is more readily seen in the spores of degenerate colonies which have been rendered atypical by artificial methods of cultivation.

Much of the confusion existing in the literature concerning the extreme variability of the actinomycetes is due to lack of recognition, or forgetfulness, of their peculiar habits of growth. The ordinary actinomycete colony (*Cohnistreptothrix* Group I Ørskov, *Streptomyces* Waksman) is not a colony in the bacterial sense; i.e. it is not an assemblage of disparate cells issuing from one or more similar cells, but the filamentous ramifying extension of the parent cell or cells. Moreover, when mature, it is composed of two phases: the vegetative substratum mycelium, and the aerial sporogenous mycelium. Recent work by von Plotho (1940), Klieneberger-Nobel (1947), and Erikson (1947*a*), has confirmed the existence of constitutional differences between these phases, already suggested by the studies of Ørskov (1923). It follows, therefore, that in a discussion of variation, such as the frequent phenomenon of asporogenous sectors produced within a single colony, it is essential to consider the origin of the inoculum in every instance. With very few exceptions, this has not been done by students of this particular problem.

Actinomyces coelicolor was selected as the test organism for this study for the following reasons. (1) Like many other saprophytic actinomycetes, it readily produces asporogenous sectors or discrete sporeless colonies on certain media; (2) it is strikingly pigmented, the range of colours varying with the pH achieved by the growth of the organism (Oxford, 1946; Cochrane & Conn, 1947) and therefore yielding a partial index of metabolic activity; (3) some strains are agar-liquefying (Stanier, 1942), which constitutes a third variable character.

The strains examined were isolated from Rothamsted soils, and include some of those (U1010, BCA1) studied by Oxford for pigment production. Some of the agar-liquefying strains described by Stanier were obtained through the courtesy of Dr van Niel. Of these the strain Waksman 3443 (W. 3443) was used for detailed study.

MEDIA AND METHODS

Simple defined media were employed throughout. Routine plating was done on Czapek's sucrose-nitrate agar. Other media used were the starch-tryptone agar of Waksman, and a substrate containing the Czapek inorganic salts plus ammonium lactate (1 %) or ammonium acetate (0·5 %) as the sole source of nitrogen and carbon. All were adjusted to pH 7·0. Sterile soil moistened with distilled water was used for maintenance of the strains. The platinum loop was used for mass transfers of vegetative and aerial mycelia. From a densely sporulating colony a loopful may contain an enormous number of viable spores, as is shown by the following experiment.

From a 7-day-old culture on starch tryptone, which enhances sporulation, six different loopfuls of aerial mycelium were taken and suspended in separate 5 ml. portions of sterile saline, run out in serial dilutions, and plated. In the 1/343,000,000 dilution one colony appeared on each of five plates, and three colonies on the sixth. Owing to the difficulty of making homogeneous suspensions of the not easily wettable spores (Erikson, 1947*a*), this does not necessarily mean that every loopful contained as many million spores as the numbers imply.

For small inocula, finely drawn-out capillary pipettes were used for seeding the plates with droplets of spores; and to the suspending fluid was added 0·1 % of a commercial detergent, which had been proved to have no toxic effect on viability. From time to time in the course of the experiments, such droplets were incubated in moist chambers with a drop of nutrient broth, and the number of developing spores observed microscopically. The number varied from 1 to 20 or even more, according to the strain, the age of culture, and medium. Despite vigorous shaking of the suspension, it was sometimes found that conidial chains remained intact. Simultaneous germination of two, three, or more numbers of such chains of varying length was observed in 90 cases, as against 893 instances of germination of detached single cells.

It is clear, therefore, that even with these precautions there can be no guarantee that the colonies on one plate are derived from a similar number of elements. Later, with the kind assistance of Dr de Fonbrune of the Pasteur Institut, Garches, the sister-spores of separate chains were isolated by means of his pneumatic micromanipulator (de Fonbrune, 1937).

RESULTS

Vegetative mycelium as inoculum

In the course of 3 years' continuous subculture and observation of 10 different isolates of *A. coelicolor*, occasional smooth sectors (i.e. apparently devoid of aerial mycelium) were seen many times in colonies on the routine media. Such vegetative growth was dissected and streaked across fresh plates, and in the majority of instances typical growth with abundant aerial mycelium occurred, if not in the first, then in the second, subculture. The most striking example was that of a culture of strain U 1010, which on Czapek glucose-nitrate agar produced an abnormal smooth orange growth, resembling that of a pro-*Actinomyces*. This strain was growing in a flask of sucrose-alanine medium, and failed to produce the soluble blue pigment. When this seemingly pure vegetative growth was streaked on Czapek sucrose-nitrate agar, it grew as characteristic colonies bearing grey-white, and occasional drab fawn sectors of aerial mycelium, and produced a limited amount of blue pigment. Further subculture yielded uniform typical growth. On careful microscopical examination of the original smooth orange growth, however, it was found that there were occasional aerial hyphae, undivided into spores, often no larger than minute buds; a development exactly similar to that which can be seen in many of the pro-actinomycetes (cf. Jensen, 1932).

To secure an inoculum in the purely vegetative phase, the aerial mycelium of cultures of each of the 10 isolates was washed off with phosphate buffer from a 7-day growth on cellophan over starch-tryptone agar, to minimize the effect of initial medium; and then dried on sterile cover-slips 9 days *in vacuo*, to obtain survival of spores only. The resulting spores were subcultured in nutrient glucose broth, where they fell from the cover-slip to the bottom of the tube and produced vegetative mycelia in 3–5 days. This culture was subcultured in the same medium every few days for 5 months; this constant subculturing in tall tubes containing 10 ml. of protein-containing media favours profuse growth which, since it is not allowed to reach the surface, is unable to produce aerial mycelium. Parallel sets of nutrient glucose broth cultures were left unsubcultured for the 5-month period.

Of the 10 frequently subcultured strains in the vegetative phase, seven produced uniform characteristic growth on plating on Czapek nitrate sucrose agar. Strain 14 yielded 24 colonies of which one produced only the yellow precursor to the red insoluble pigment. The progeny of this colony were typical blue and yellow colonies in about equal numbers. Strain Ph-C gave 20 small colourless colonies, five large typical blue colonies, and one small purple one, Strain pH-R 2 was similar, but four of the small colonies had red pigmented sectors. All bore more or less abundant aerial mycelium. On further subculture none of the variants proved to be stable.

When the 5-month-old cultures were examined, only four of the strains still showed bottom vegetative growth. The others had some surface aerial mycelium. Subcultured on various plate media, each strain produced a high proportion of non-pigmented or yellow to reddish purple colonies, often with sectors and without aerial mycelium. One smooth red colony from strain pH-R (1)*b* was selected for detailed examination. For the first two subcultures it produced a mixture of typical pigmented sporing colonies and of colourless or reddish sporeless colonies. Sowing from these simultaneously, on the two halves of a plate, each of these two types then bred true on all defined media for over a year. But microscopical observation of almost any of the apparently smooth colonies revealed that there were some aerial filaments, usually undivided, just as in the orange growth of U 1010 noted above. The loss of the power to produce aerial mycelium was not complete.

This was finally proved by growing the non-sporing variants in sterile soil for 2–4 weeks. Crumbs of the inoculated soil became covered with typical aerial spirals subdivided into spores, and on replating this property was retained. The power of pigment production, however, was not usually restored by passage through soil. Nevertheless, this could not be regarded as entirely lost. Occasional bacterial and fungal contaminants producing respectively an alkaline and an acid pH, induced blue and red coloration of the actinomycete growth in their respective vicinities. The colourless colonies had evidently elaborated the precursor to the pigment, although they were unable by their own metabolic activity to establish the requisite pH for its manifestation.

The vegetative bottom growth from a variety of other media was also tested for uniformity on the routine solid media. These media, adjusted to pH 7·0 contained ammonium phosphate as the source of N, the usual Czapek inorganic salts, and 1 % by weight of each of the following substances: sucrose, glucose, lactose, fructose, maltose, glactose, arabinose, raffinose, xylose; mannitol, sorbitol, dulcitol; salicin, inulin, starch; tartaric, succinic, uric, citric, malic, acetic, lactic acids; cellulose (not weighed) in the form of strips of pure Swedish filter-paper. Xylose, dulcitol, tartrate, and citrate did not support growth. From all media that supported good growth, characteristic uniform colonies were produced on plating. Where the growth was poor, as with arabinose, malate, and acetate, there was a tendency towards sectoring. All strains behaved more or less similarly.

The effect of a high concentration of Ca ion ($CaCl_2$, 25 g./l.) was also tested in the nitrate-sucrose medium recommended by Foster, McDaniel, Woodruff & Stokes (1945) for the production of fruiting bodies in submerged cultures of *P. notatum*. In this substrate growth was poor, and the filaments showed an abnormal degree of condensation of the protoplasm in densely staining blocks, as well as thickening of portions of the cell walls. Yet on plating on starch-tryptone agar typical colonies were obtained.

Aerial mycelium as inoculum

Commencing with the same material as was used for the experiments in liquid media, namely, dried washed spores on cover-slips, plates were seeded with droplets of the spore suspension and subcultured regularly during the 5-month period. The same general phenomena were observed. The dominant colony was that characteristic of the species: it bore spirally coiled aerial mycelium abundantly divided into spores, produced a red insoluble pigment in the vegetative mycelium and a blue soluble pigment that diffused into the medium as it became alkaline. From time to time many variants were observed on the plate; colourless, pale pink, red, purple, with yellow, orange or green reverse; varying greatly in size, and having no aerial mycelium or very little, mostly not divided into spores. On subculture these variants frequently produced similar mixtures, but also some of the dominant colonies. Mass transfers of typical growth usually yielded uniform growth on plates.

Monoconidial isolates

Micromanipulator isolations were made from four different types of colony, two each from two different strains:

(1) A typical well-sporulated, vigorous, blue-pigmented, agar-liquefying, colony of W. 3443.

(2) A poorly sporulated, yellow-pigmented, sectored, non-agar-liquefying colony of W. 3443; of the same age, and on the same medium as (1).

(3) A well-sporulated, vigorous, yellow-pigmented colony of the variant Ph-R (1)*b*, which for a year had been kept almost entirely sporeless and colourless, but which after passage through sterile soil had recovered the power of producing aerial mycelium, and to a slight extent that of pigment production

(4) A poorly sporulated, colourless, sectored colony of Ph-R(1)*b*, of the same age, and on the same medium as (3).

The spores were incubated in droplets of nutrient glucose broth in the oil chamber of Comandon & de Fonbrune (1938). Not all were viable. There were also observable differences in the rate of germination. From (1), three chains, 1/1, 1/2, 1/3, were isolated and the sister-spores separated and cultured simultaneously under identical conditions:

1/1: six spores; two (*a* and *b*) viable;
1/2: eight spores; eight (*a*–*h*) viable;
1/3: eight spores; five (*a*–*e*) viable.

When seeded on Czapek nitrate-sucrose agar for ten generations, the following results were obtained:

1/1*a*: uniform characteristic growth;

1/1*b*: uniform growth, but only yellow pigment, no agar liquefaction;

1/2*a*: uniform characteristic growth;

1/2*b*: a colourless, non-agar-liquefying variant obtained, breeding true for 30 generations (Pl. 1, fig. 1–3);

1/2*c*, 1/2*d*, 1/2*e*, 1/*f*: all uniform characteristic growth;

1/2*g*: a variant similar to 1/2*b*;

1/2*h*: uniform characteristic growth;

1/3*a*–1/3*e*: all five spores yielded uniform characteristic growth.

Thus, of the 15 strains derived from spores taken from three separate chains from a well-grown colony, 12 produced the characteristic growth, and three had lost the power to produce pigment and to liquefy agar.

From colony 2, two chains were isolated:

2/1: twelve spores; two (*a* and *b*) were viable;
2/2: eight spores; one (*a*) was viable.

All three isolates tended strongly to grow in sectored colonies with loss of pigmentation, incomplete sporulation, and inability to liquefy agar. Ultimately, two separate variants, corresponding to those shown in Pl. 1, fig. 1, were obtained from 2/1*a*.

From colony 3, two chains were isolated:

3/1: eight spores; six (*a–f*) were viable;
3/2: eight spores; eight (*a–h*) were viable.

Only one, 3/2*f*, of the 14 single-spore strains varied, producing colourless, sporeless, and blue-pigmented sectors. The rest produced good aerial mycelium and varying degrees of yellow pigmentation on the reverse of the colony, as in the parent culture.

From colony 4, two chains were isolated:

4/1: eight spores; four (*a–d*) were viable.
4/2: four spores; one was viable.

All five isolates yielded poor growths with a marked tendency towards sectoring; colourless, with sparse aerial mycelium; with yellow to reddish pigment, and irregular aerial mycelium. In no instance was the typical, well-sporulated, blue-pigmented colony obtained.

Sector production

The suggestion that multiple sectored colonies arise from more than one conidium, which has frequently been made (Stanier, 1942), has not been substantiated. This is shown in Pl. 1, fig. 4; *A* was seeded with the colourless, non-agar-liquefying variant 1/2*b*, and *B* with a culture typical of the parent strain W. 3443, both derived from the same single spore. The central portion was seeded with a mixture of spores from 1/2*b* and the typical growth. The appearance of the mixed colonies was the same as that of the typical growth, as regards aerial mycelium, pigment production, and a slight degree of agar liquefaction. Multiple colonies could be seen, but they were uniform, not sectored. Further experiments were made, growing 1/2*b* and the typical strain separately and mixed, in liquid media and in soil, and then plating both mixed suspensions of spores from the separate growths and suspensions of spores from the mixed growth; the resultant colonies could not be distinguished from the dominant typical colonies.

DISCUSSION

If we disregard all the temporary variants manifested by the different shades of pigmentation and varyingly efficient degrees of sporulation, the main variable characters to be considered are pigment, aerial mycelium and agar liquefaction.

Complete loss of pigmentation was maintained during 1 year's frequent subculturing with strain Ph–R(1)*b*, derived from the vegetative mycelium of a 5-month-old broth culture. Simultaneously, the power of producing aerial mycelium was so very greatly diminished, that to the naked eye the mycelium appeared to be sporeless.

Vegetative mycelium in a vigorously growing state produced only occasional variants with temporary loss of these two attributes, whatever the liquid medium in which it was grown. It is clear, therefore, that it is the degraded condition and diminution of vigour induced by artificial means which bring about the variation by loss. This is confirmed by microscopical examination of such aged mycelium, which shows an extraordinary degree of beading, segmentation, and vacuolation of the filaments, and only very small portions of which proved to be viable; and by the fact that passage in soil, a natural habitat, restores the property of producing aerial mycelium, and to a slight extent that of pigmentation. Normally, then, the vegetative mycelium reproduces its kind.

Analysing the results of culturing the single spores of two different types of colony in two separate strains, we find that a higher proportion of spores were viable and their progeny more stable in the case of the two vigorous, well-sporulated colonies: 1 (W. 3443), and 3 (Ph-R(1)*b*). The less well-developed parent colonies, 2 (W. 3443) and 4 (Ph-R(1)*b*), which already showed diminution in aerial mycelium production and little or no pigmentation, yielded a lower proportion of viable spores whose progeny was marked by sectoring and instability. This accords with expectation, but in the time available the numbers of single spores isolated were not large.

Nevertheless, variation can occur within a homogeneous population in a vigorous state of growth. Thus, in the variant 1/2*b*, the typical pigmented, agar-liquefying colonies on one half of Pl. 1, fig. 1 are strikingly different from the equally vigorous, even larger, colourless, non-agar-liquefying colonies on the other half. Yet all are the progeny of one spore. There is also a difference in the degrees of brittleness and non-wettability of the aerial mycelium in each case, the non-pigmented variety being more easily detached and considerably more resistant to the action of wetting agents (see Erikson, 1947*b*; also Velu, Comandon, de Fonbrune, Janot, Penau, Mainil & Bouet, 1947, on monoconidial isolates of *P. notatum*). Six of the sister-spores of the same chain produced only the typical growth. One must conclude that there is an inherent tendency for a given mutation to occur more frequently in one clone than another.

The linkage of pigmentation with agar-liquefaction is not quite complete. Passage of the colourless 1/2*b* in soil restored a very slight degree of pigmentation (occasional yellow-green patches), but not that of agar-liquefaction. As Braun (1947) has pointed out, many linked characters can be attributed to one basic change only; or to a single mutation at different levels of gene-controlled processes; or to the influence of one mutation on the rate of occurrence of another mutation. The mechanism is not yet clear.

The tendency of some spores to produce colourless variants is commonly masked by the pigmentation of the dominant parent strain, as is shown by the experiments with the mixture of spores from the two types of 1/2*b*. Schaal (1944) also has illustrations of colourless and pigmented variants from a single spore of *Actinomyces scabies*.

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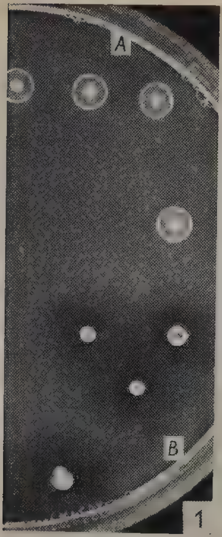

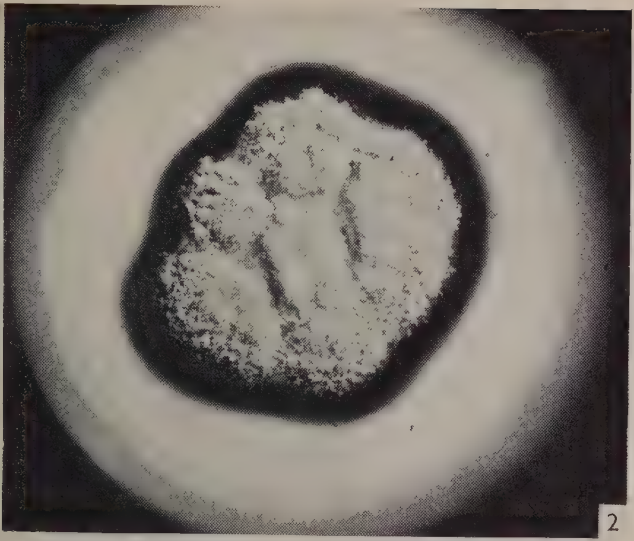

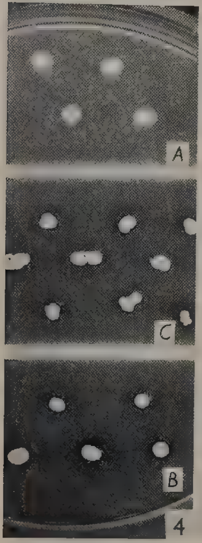

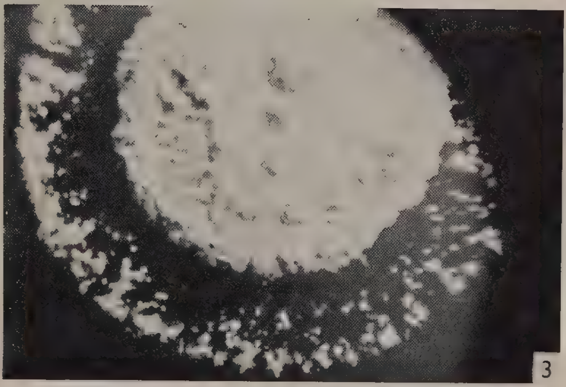

Figs. 1–4

D. ERIKSON—VARIATION IN *A. COELICOLOR*. PLATE 1

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EXPLANATION OF PLATE

Fig. 1. The two variants of 1/2*b* derived from one spore; (*A*) colourless, non-agar-liquefying; (*B*) blue-pigmented, agar-liquefying.

Fig. 2. Discrete colony from (*B*) in Fig. 4; the aerial mycelium is colourless, the substratum mycelium is red, and a ring of soluble blue pigment has diffused into medium. × 16.

Fig. 3. Part of a discrete colony from (*A*) in Fig. 4; showing different disposition of colourless aerial mycelium.

Fig. 4. The two variants of 1/2*b*, (*A*) and (*B*) shown in Fig. 1, and (*C*) a mixture of the two.

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The Action of Lysozyme on Heat-killed Gram-positive Micro-organisms

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SUMMARY: The change in the Gram staining reaction which occurs when heat-killed Gram-positive *Clostridium welchii* and *Staphylococcus albus* are incubated with lysozyme is due to the removal of the ribonucleic acid component of the Gram complex, and is brought about by the hydrolysis of certain sugar linkages in polysaccharides located at the cell surface.

Lysozyme, the enzyme in egg-white which lyses living suspensions of susceptible saprophytic micrococci, particularly *Micrococcus lysodeikticus*, has been the subject of numerous investigations since its discovery by Fleming in 1922. These have been reviewed by Mesrobeanu & Noeppel (1938) and by Thompson (1940). The enzyme is a basic protein or polypeptide of molecular weight 18,000–25,000 containing about 16 % nitrogen and 2–3 % sulphur (Abraham, 1939; Thompson, 1940). The isolation and purification of lysozyme has recently been improved by a procedure which includes direct adsorption of the enzyme from native egg-white on to bentonite, followed by elution with 5 % aqueous pyridine adjusted to pH 5 with sulphuric acid (Alderton, Ward & Fevold, 1945).

The first attempt to study the action of lysozyme was made by Meyer, Palmer, Thompson & Khorazo (1936). Unfortunately, the authors used an enzyme prepared from a *Sarcina* sp. susceptible to egg-white lysozyme and which had properties similar to the latter enzyme, but which might have contained an autolytic enzyme system of the *Sarcina*. The enzyme had a specific action on the sugar linkages of certain carbohydrates containing amino sugars, thus supporting an original suggestion of Hallauer (1929). This finding, however, may not apply to lysozyme of egg-white. Epstein & Chain (1940) isolated a polysaccharide fraction from the cell bodies of *M. lysodeikticus* and other sensitive bacteria which was hydrolysed by lysozyme with production of N-acetyl hexosamine. The polysaccharide was found in all organisms sensitive to lysozyme, the largest amount being present in *Bacillus subtilis*, an organism which was killed, but not lysed, by lysozyme. Meyer & Hahnel (1946) confirmed the fact that lysozyme hydrolysed a carbohydrate fraction isolated from sodium hydroxide extracts of the susceptible organisms. Meyer (1946) explained lysozyme action in terms of the hydrolysis of a mucopolysaccharide in the bacterial membrane. This mucopolysaccharide, which appears to be firmly bound to the bacterial membrane, is first depolymerized by lysozyme with consequent water imbibition by the organism and disorganization of the cell. Autolytic enzymes, active after the death of the organisms, were considered to be responsible in part for the clearing of the bacterial suspension.

It is possible that heat inactivation of the autolytic enzyme systems may be of greater significance in explaining the resistance of killed cells to complete lysis by lysozyme than the fact that cellular proteins are rendered insoluble on heating (cf. Fleming & Allison, 1922). Lysis of bacteria by lysozyme is preceded by the conversion of the Gram-positive cells to Gram-negative forms (Meyer, 1946); this also occurs when suspensions of heat-killed cells are incubated with lysozyme. Thompson & Dubos (1938) showed that the first stage of the autolysis of a rough Type II pneumococcus, namely its conversion from Gram-positive to Gram-negative without disintegration of cellular structure, was accompanied by the liberation of a ribonucleoprotein and ribonucleic acid. More recently, Henry & Stacey (1946) have shown that suitable Gram-positive cells can be rendered Gram-negative by extraction with sodium cholate solution at 60°. The bile-salt extract contained inert carbohydrate, traces of protein and lipids, and magnesium ribonucleate. The residual Gram-negative cells, after treatment with suitable reducing agents such as dilute formaldehyde in 0·85 % saline, could be recombined with the magnesium ribonucleate extracted from the same and other micro-organisms, to become Gram-positive again. It was also shown (Henry, Stacey & Teece, 1945) that a ribonucleoprotein was released during a relatively short autolysis of *Clostridium welchii.*

Enzymes have been obtained from pancreas (Kunitz, 1940) and from leucocytes (Dubos & MacLeod, 1938) which convert killed Gram-positive cells to the Gram-negative state; these enzymes have in common the property of hydrolysing ribonucleic acid. Furthermore, one of the autolytic enzymes of Gram-positive micro-organisms which converts the cells to Gram-negative forms also has this property (Jones, Stacey & Webb, 1948). The results of Spanier & Deribas (1937), in which it was claimed that egg-white lysozyme contained a nucleotidase active against yeast nucleic acid and which conditioned the bacteriolytic properties of the enzyme, therefore attain a greater significance. These results, which do not appear to have been investigated further, were unfortunately obtained with impure enzyme preparations which hydrolysed with liberation of inorganic phosphate.

In the light of the more recent information concerning the structure of the Gram-positive complex, and of the nature of enzymes capable of affecting the conversion of Gram-positive cells to Gram-negative forms, it was of interest to examine the reactions which occur when such a change is brought about by the action of lysozyme on killed cells.

EXPERIMENTAL

Preparation of lysozyme

Lysozyme was isolated from egg-white according to the method of Alderton *et al.* (1945). Owing to the scarcity of the starting material no attempts were made to crystallize the enzyme. The activity of the preparations, as determined by the method of Goldsworthy & Florey (1930) against *M. lysodeikticus*, ranged between 5000 and 6000 lysozyme units/mg. The enzyme solution used contained 1 mg. of the lysozyme/ml.

Action of lysozyme on heat-killed Gram-positive organisms

Preparation of heat-killed cells. Cultures of *Cl. welchii* were grown anaerobically in 2 % (w/v) peptone-glucose broth at 37°. The cells were harvested in a Sharples centrifuge after 18 hr. incubation. *Streptococcus faecalis* and the strains of staphylococci and micrococci were grown on 2 % peptone agar in Roux bottles at 37° and were removed in distilled water after 24 hr. The strains of *Staphylococcus aureus* used were recently isolated from human pathological specimens; the micrococci were saprophytic organisms isolated from the air and skin.

The centrifuged organisms were washed with distilled water, resuspended in distilled water and killed by heating at 80°. Killed cells, especially those of *Cl. welchii* prepared by simply immersing a suspension of the Gram-positive bacteria in a water-bath at 80° for 30 min., were unsuitable since stained smears revealed the presence of a variable percentage of Gram-negative forms. The procedure finally adopted was the 'flash sterilization' method of Dubos & MacLeod (1938) in which a small volume of bacterial suspension is rapidly added to a large volume of distilled water at 80° After 30 min. at this temperature the suspension is cooled, centrifuged and the cells resuspended in distilled water.

The fact that Gram-positive cells in aqueous suspension readily pass to the Gram-negative state when heated at 80° suggests that the components of the Gram-positive complex may be loosely bound. In general, the quantity of lysozyme required to convert heat-killed Gram-positive cells to the Gram-negative state was considerably greater than that required to lyze living suspensions of *M. lysodeikticus*. The method finally adopted was as follows.

A suspension of the killed cells was added to lysozyme solution (0·5 ml.) 0·2 M phosphate buffer pH 7·0 (1·0 ml.) and physiological saline (3·5 ml.) so that the final opacity approximated to no. 10 on McFarland's (see Kolmer, 1925) standard barium sulphate scale. The same volume of the cell suspension was added to a control tube containing 0·2 M phosphate buffer pH 7·0 (1·0 ml.) and physiological saline (4·0 ml.). Toluene (0·1 ml.) was used as preservative since it has no effect on the activity of lysozyme (Fleming, 1922). The tubes were corked and incubated at 37°. After appropriate periods, the suspensions were centrifuged and duplicate smears stained by the Gram method; the approximate percentage of Gram-negative cells was estimated visually.

The results (Table 1) show that all the killed Gram-positive organisms, with the exception of yeast, became more or less completely Gram-negative. Suspensions of *Strep. faecalis* and *Staph. aureus* (Thompson) were also caused to flocculate. A similar effect, which has been attributed to adsorption of the enzyme by the bacterial cells and to their particular colloidal structure, has been observed with suspensions of living organisms (Friedberger & Hoder, 1932; Klemparskaya, 1939). The Gram-negative cell bodies remaining after the action of lysozyme were in all cases readily dissolved by trypsin which was without effect on the intact Gram-positive organisms.

Since saprophytic bacteria are, in general, regarded as being more susceptible than pathogenic organisms to lysis by lysozyme (Mesrobeanu & Noeppel, 1938; Thompson, 1940), it is of interest that the heat-killed cells of the pathogenic strains of *Staph. aureus* were as susceptible to the action of lysozyme as were the saprophytic micrococci. Furthermore, strains of staphylococci reported to be resistant to the action of lysozyme, i.e. *Staph. albus* (Epstein & Chain, 1940; Thompson & Khorazo, 1935) and *Staph. aureus* (Thompson & Khorazo, 1935), were as susceptible in the killed state to the action of lysozyme as the killed cells of the readily lysed *Staph. citreus* (Thompson & Khorazo, 1935).

Table 1. *Activity of lysozyme in converting heat-killed Gram-positive cells to Gram-negative forms at* pH 7·0

Organism	Duration of exp. (hr.)	Approx. % Gram-negative cells: Lysozyme-treated cells	Approx. % Gram-negative cells: Control
Cl. welchii (S)	24	100	30
Cl. welchii (R)	24	80–100	0
Strep. faecalis	72	100*	20
Staph. citreus (B9)	72	80	20
Staph. albus (9238)	24	100	20
Staph. aureus (Thompson)†	72	80*	20
Staph. aureus 482†	96	100	30
361†	96	100	30
709†	96	70–80	30
395†	96	100	50
Micrococcus sp. 475	96	50–60	10
446	96	100	0
470	96	100	0
467	96	70	0
Saccharomyces cerevisiae	72	0	0
	129	0	0
	168	0	0

* Flocculation. † Pathogenic strains

The pH optimum for the action of lysozyme on heat-killed Clostridium welchii *and* Staphylococcus albus

The lysozyme solution (0·5 ml.) in physiological saline (3·5 ml.) was buffered to various pH values with 0·2 M acetate (pH 2–6) or 0·2 M phosphate (pH 7–10) solutions (1·0 ml.). A uniform, washed suspension of the heat-killed Gram-positive organisms (0·5 ml.) was added to each tube of the series. After 48–72 hr. incubation the suspensions were centrifuged and duplicate stained smears prepared. The results (Table 2) show that with each organism the conversion to the Gram-negative state was optimal at pH 6. This value agrees closely with that (pH 6·2) determined by means of the Moll extinctiometer as the optimum for the lysis of suspensions of *M. lysodeikticus* by the enzyme (Boasson, 1938).

The action of lysozyme on ribonucleic acid

In view of the claims of Spanier & Deribas (1937) that lysozyme contains a nucleotidase, and of the fact that enzymes which hydrolyse ribonucleic acid also convert killed cells from Gram-positive to Gram-negative, lysozyme preparations were examined for ribonucleinase activity.

Lysozyme preparations (0·5 ml.) were examined for hydrolytic activity against 0·1 % (w/v) sodium ribonucleate (2·0 ml.) in 0·2 M acetate buffer pH 6·0 (1·5 ml.) by the method of Davidson & Waymouth (1944). The unchanged ribonucleic acid precipitated by MacFadyen's uranyl acetate-trichloroacetic acid reagent after incubation with the enzyme was centrifuged down after 6 hr., washed with 0·125 % (w/v) uranyl acetate in 1·25 % (w/v) trichloroacetic acid and analysed for total phosphorus by Allen's (1940) colorimetric method.

Table 2. *Variations with* pH *in the action of lysozyme on heat-killed Gram-positive* Clostridium welchii (*S*) *and* Staphylococcus albus 9238

pH	Approx. % Gram-negative cells	
	Cl. welchii	*Staph. albus*
2·0	10	30
3·0	20	30
4·0	30	50
5·0	70	100
6·0	100	100
7·0	90–100	90–100
8·0	90	90
9·0	90*	80
10·0	50–60*	60

Duration of experiment 48–72 hr.
* Cells gelatinous in character and emulsified with difficulty.

Table 3. *Examination of lysozyme for ribonucleinase activity*

Time of incubation at 37° (hr.)	Phosphorus content (mg.) of ribonucleic acid precipitated after incubation with lysozyme preparations						
	B1	A1	A2	A4	E5	L4	L5
0	0·083	0·048	0·048	0·050	0·087	0·052	0·035
1·0	—	0·047	0·046	0·058	—	—	—
1·5	0·083	—	—	—	0·086	—	—
2·0	—	0·050	0·046	0·049	—	0·053	0·034
3·0	0·090	0·046	0·046	0·049	—	—	—
3·5	—	—	—	—	0·087	—	—
4·5	—	0·050	0·045	—	—	—	0·038
5·0	0·082	—	—	—	0·085	—	—
6·0	—	0·045	0·045	0·049	—	0·049	—
6·5	0·084	—	—	—	—	—	—
8·0	0·088	—	—	—	—	0·051	0·035
24·0	—	—	—	—	0·088	—	—

The colours were compared in the Spekker adsorption photometer, using Ilford 608 filters. The results (Table 3) show that lysozyme has no ribonucleinase activity.

Liberation of reducing sugar during the action of lysozyme on heat-killed cells

Although only relatively small increases in reducing sugar were obtained in these determinations, the Schaffer-Hartmann (1920) method of analysis used in conjunction with the recently described starch-glycollate indicator (Peat, Bourne & Thrower, 1947) gave accurate and reproducible results. Somogyi's (1937) modification proved unsatisfactory, as changes occurred in the standard alkaline copper solution on standing.

Control experiments showed that no liberation of reducing substances occurred when lysozyme solutions or suspensions of killed cells were incubated alone. When, however, suspensions of killed cells were incubated with lysozyme until Gram-negative, small amounts of reducing sugar were liberated (Table 4) as shown by the following experiments.

Staph. albus. The cells from 20 Roux bottles of a 24 hr. culture of the organism (≃2·5–3 g. dry cells) were removed in distilled water and killed by the 'flash-sterilization' method. The washed cells in distilled water (50 ml.) were added to 0·2 M acetate buffer pH 6·0 (60 ml.) and lysozyme (45 ml.) and the resulting suspension incubated at 37° in the presence of toluene (5 ml.). At suitable intervals during the change from Gram-positive to Gram-negative samples of the suspension were withdrawn and centrifuged at high speed (6000 r.p.m.) until the supernatants were clear and free from cells. The reducing sugar (as glucose)/5 ml. (Table 4) in each solution was then determined by the Schaffer-Hartmann method.

Table 4. *Liberation of reducing sugar during the conversion of heat-killed Gram-positive* Staphylococcus albus *and* Clostridium welchii *to Gram-negative forms by lysozyme*

	Total reducing sugar (mg. as glucose) liberated during the action of lysozyme on heat-killed		
Time (hr.)	*Staph. albus*	*Staph. albus*	*Cl. welchii*
0	0·00	0·00	0·00
21·5	—	—	2·36
22·0	0·84	—	—
45·0	—	2·35	—
47·0	1·95	—	—
70·0	—	2·72	—
93·0	3·57	—	—
100·0	—	—	4·01
126·0	3·96	—	—
173·0	4·74	—	—

Cl. welchii. The cells from 600 ml. of an 18 hr. broth culture (≃0·3 g. dry cells) were collected (centrifuge), washed with distilled water and killed by the 'flash-sterilization' method. A suspension of the cells in distilled water (30 ml.) was added to lysozyme (5 ml.) in 0·2 M acetate buffer pH 6·0 (15 ml.) and incubated at 37° in the presence of toluene (2 ml.). Analyses for reducing sugar, made during the conversion from Gram-positive to Gram-negative as described above, yielded the results recorded in Table 4.

Action of lysozyme on the specific carbohydrates of Staphylococcus citreus (*B* 9) *and* Clostridium welchii

The carbohydrates used were crude preparations isolated by procedures which, in the case of the staphylococcus, did not destroy the Gram staining properties of the organism, and, in the case of *Cl. welchii* did not destroy the cellular structure, although the Gram reaction became negative. The polysaccharides may, therefore, be considered as surface components of the bacterial cells.

The carbohydrate from Staphylococcus citreus (*B* 9). Of the several methods investigated for the isolation of the polysaccharide, namely extraction with

0·05 N sodium hydroxide at 60°, 0·06 N hydrochloric acid at 100° for 30 min. (Julianelle & Wieghard, 1935), 2 % sodium cholate at 60° and distilled water at 100° for 5 hr., the last was found to give the best results. The procedure was as follows:

The growth from 20 Roux bottles of a 48 hr. culture of *Staph. citreus* (≏ 2·5–3 g. dry cells) was removed in distilled water (200 ml.). The suspension was filtered through glass-wool and then centrifuged. The cells were washed twice with distilled water, suspended in distilled water (100 ml.) and heated for 5 hr. at 100°, cooled and centrifuged. Acetic acid (5 N) was added to the supernatant to bring to pH 3·5 and the resulting precipitate removed (filtration) after 2 hr. Sodium acetate (1 g.) was dissolved in the filtrate and the crude polysaccharide precipitated by the addition of ethanol (4 vols.). After 24 hr. the precipitate was collected (centrifuge), suspended in distilled water (50 ml.) and 50 % (w/v) trichloroacetic acid (5 ml.) added. The precipitate formed was removed (filtration) after 30 min. and the carbohydrate reprecipitated from the filtrate by the addition of ethanol (4 vols.). After 48 hr. the precipitate was collected (centrifuge), dissolved in distilled water (40 ml.) and the above process repeated until no further material was precipitated by the addition of trichloroacetic acid. The solution was then dialysed against tap water (3 × 1000 ml.) for 48 hr. and filtered. Sodium acetate (1 g.) was dissolved in the filtrate and the polysaccharide then precipitated by the addition of ethanol (5 vols.). The precipitate was collected (centrifuge) after 48 hr., washed with ethanol and dried with ethanol and ether.

The cells from a total of 120 Roux bottles yielded 400 mg. of polysaccharide as a white powder having $[\alpha]_D = +16°$ in water ($c = 1·0$). Aqueous solutions gave a negative biuret reaction and were non-reducing before hydrolysis.

The carbohydrate from Clostridium welchii. The cells from 10 l. of an 18 hr. culture of *Cl. welchii* (≏ 5 g. dry cells) were washed with distilled water and suspended in 0·05 N sodium hydroxide (100 ml.). The suspension was maintained at 60° for 18 hr. and then centrifuged. Acetic acid (5 N) was added to the clear supernatant to bring to pH 3·5 and the resulting precipitate removed by filtration after 2 hr. The crude polysaccharide precipitated from the filtrate by the addition of ethanol (4 vols.) was purified by repeated fractionation with trichloroacetic acid as described above. Finally, the polysaccharide was precipitated with ethanol (5 vols.), washed with ethanol and dried with ethanol and ether.

The cells from 40 l. of culture medium yielded 313 mg. of the specific polysaccharide as a white powder which showed $[\alpha]_D = +51°$ in water ($c = 0·7$). The carbohydrate was readily soluble in water (concentrated aqueous solutions exhibiting high viscosity) and was precipitated from solution by copper sulphate, uranyl acetate and ferric chloride. It was non-reducing before hydrolysis. With ninhydrin a weak positive reaction was obtained, but no precipitation occurred on adding 50 % (w/v) trichloroacetic acid to the concentrated aqueous solution.

Aqueous solutions of these polysaccharide fractions adjusted to pH 6 were incubated at 37° with lysozyme in the presence of toluene. At the time intervals shown in Table 5, 5 ml. fractions were withdrawn and analysed for reducing sugar according to the Schaffer-Hartmann method. The results (Table 5) show that both the specific carbohydrate of *Staph. citreus* and *Cl. welchii* were hydrolysed to a small but definite extent by lysozyme.

Initially the carbohydrate and lysozyme solutions were completely clear and free from particles in suspension, but on incubation of the mixed polysaccharide and enzyme solutions some precipitation occurred within 24 hr. This observation may possibly have some bearing on the phenomenon of bacterial flocculation by lysozyme.

It appeared from these results that when lysozyme acts on heat-killed cells a small proportion of reducing sugar is liberated from carbohydrate material located at the surface of the cells. In support of this, it was found that reducing substances were not liberated by the action of lysozyme on *Cl. welchii* cells which had been rendered Gram-negative by extraction with sodium cholate (Henry & Stacey, 1946), a process which is known to be accompanied by the loss of ribonucleic acid and carbohydrate from the cell surface.

Table 5. *Hydrolysis of the specific carbohydrates of* Staphylococcus citreus *and* Clostridium welchii *by lysozyme at* pH 6

	Mg. reducing sugar (as glucose) liberated/100 mg. polysaccharide from			
Time (hr.)	*Staph. citreus**	*Staph. citreus**	*Cl. welchii**	*Cl. welchii†*
0	0	0	0	0
24	1·39	1·50	1·65	0·68
50	1·42	—	2·00	0·74
120	—	1·59	—	—
164	1·42	1·75	2·17	1·16

* 60 mg. polysaccharide in distilled water (13 ml.) with lysozyme (2 ml.) in 0·2 M acetate buffer pH 6·0 (10 ml.).

† 110 mg. polysaccharide in distilled water (12 ml.) with lysozyme (3 ml.) in 0·2 M acetate buffer pH 6·0 (5 ml.).

Since the conversion of Gram-positive cells to Gram-negative forms is known to involve the removal of ribonucleic acid from the cell surface, it appeared from the experimental results thus far obtained that this change must occur concurrently with the hydrolysis of surface carbohydrate substances which takes place when killed cells are rendered Gram-negative by the action of lysozyme. That this is the case is proved by the following experiments in which fractions were isolated from the material liberated by the action of lysozyme on heat-killed *Staph. albus* and *Cl. welchii* and which, in each case, had the properties of a ribonucleic acid.

Isolation of ribonucleic acid from the material liberated from heat-killed Staphylococcus albus *and* Clostridium welchii *during the lysozyme-induced change from Gram-positive to Gram-negative*

Staph. albus. A suspension of heat-killed Gram-positive *Staph. albus* (9238) cells from 20 Roux bottles (≃2·5–3 g. dry organisms) in distilled water (50 ml.) was incubated with lysozyme (45 ml.) in 0·2 M acetate buffer pH 6·0 (60 ml.) and toluene (5 ml.) until the cells were completely Gram-negative (170 hr.). The suspension was then centrifuged and the crude nucleic acid precipitated from the clear supernatant by the addition of an equal volume of 0·25 % (w/v) uranyl acetate in 2·5 % (w/v) trichloroacetic acid solution. The precipitate was collected by centrifuging after standing for 18 hr., suspended in distilled water (20 ml.) and 0·5 M sodium carbonate (2 ml.) added. The insoluble material (biuret positive, Molisch negative) was removed at the centrifuge after 5 min., and the clear supernatant brought to pH 2·5 with

5 N acetic acid. After 18 hr. the precipitate (biuret negative) was centrifuged down, suspended in distilled water (15 ml.) and 0·5 M sodium carbonate added until complete solution was obtained. Acetic acid (2 N) was then added to pH 6·0 and the solution centrifuged. Lanthanum acetate (1 % (w/v); cf. Caspersson, Hammarsten & Hammarsten, 1935) was then added dropwise to the clear supernatant until no further precipitation occurred. The solid was collected at the centrifuge after 18 hr., suspended in distilled water (15 ml.) and dissolved by the dropwise addition of 0·5 M sodium carbonate. Separation of lanthanum carbonate occurred on standing. After 6 hr. the suspension was centrifuged and the precipitate washed once with distilled water containing a little sodium carbonate. The combined supernatants were adjusted to pH 2·5 with N hydrochloric acid and the resulting precipitate collected (centrifuge) after 18 hr. The deposit was washed with ethanol and dried with ethanol and ether. Yield 43 mg. of a light brown powder.

Cl. welchii. The cells from 6 l. of a 16 hr. culture of *Cl. welchii* (≏ 3·0 g. dry cells) were collected (Sharples Supercentrifuge), washed once with water, suspended in distilled water and killed at 80°. A suspension of the cells in distilled water (70 ml.) was incubated at 37° with lysozyme (50 ml.) in 0·2 M acetate buffer pH 6·0 (80 ml.) and toluene (2 ml.) until the cells were completely Gram-negative (4–5 days). The suspension was then centrifuged and the clear supernatant (180 ml.) precipitated by the addition of the uranyl acetate + trichloroacetic acid reagent (180 ml.). Purification of the precipitate according to the above method yielded 65 mg. of a light brown powder.

The preparations from *Staph. albus* and *Cl. welchii* thus obtained contained a high percentage of ash (Table 6) which was green in colour. The percentage of these inorganic substances (possibly introduced as impurities present in the lanthanum acetate) in the fraction from the staphylococcus could be diminished from 26 to 19 % by dialysis of a solution of the material in dilute sodium carbonate, but could not be removed completely.

Properties of the nucleic acid fraction from Staphylococcus albus *and* Clostridium welchii

The analytical figures (corrected for ash) found for the two preparations are recorded in Table 6 and are compared with the theoretical values calculated for ribonucleic acid of the formula $C_{38}H_{49}O_{29}N_{15}P_4$.

Table 6. *Properties of the nucleic acid fraction from* Staphylococcus albus *and* Clostridium welchii

	Nucleic acid fraction from		Calc. for ribonucleic acid
Property	*Staph. albus*	*Cl. welchii*	$C_{38}H_{49}O_{29}N_{15}P_4$
Biuret reaction	Negative	Negative	—
Millon's reaction	Negative	Negative	—
Ninhydrin reaction	Negative	Negative	—
Feulgen (deoxypentose) reaction	Negative	Weak positive	—
Dische reaction	Negative	Weak positive*	—
Ash (%)	26·0	26·8	—
N (%)	15·2	14·8	16·14
P (%)	9·2	8·7	9·64
Ratio *N*/*P*	1·65	1·7	1·69
Pentose (Bial) (%)	54·1	43·6–46·6	46·1
Pentose/phosphorus	4·9	5·0–5·35	4·84

* Corresponding to the presence of 3–5 % deoxyribonucleic acid.

Further evidence for the identity of these fractions with ribonucleic acid was provided by the following facts.

The preparations exhibited strong adsorption bands at 260·0 mμ. which, after correcting for the differences in ash content, corresponded with the adsorption band given by a solution of the same concentration of ribonucleic acid purified as described by Henry & Stacey (1946) (ash content 8·7 %).

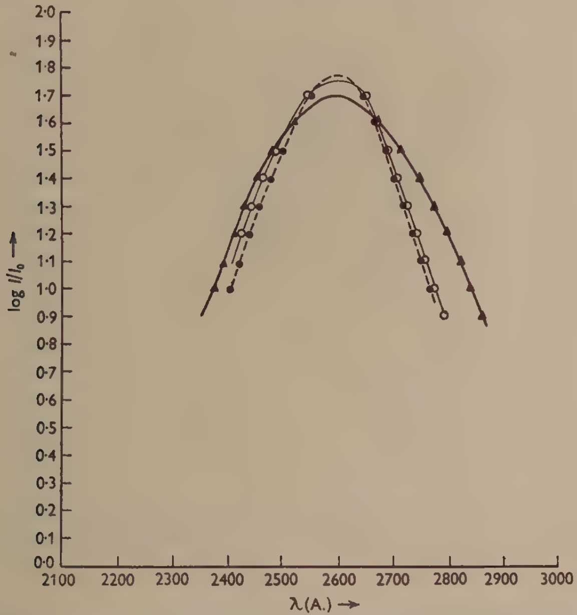

Fig. 1. Adsorption spectra of yeast ribonucleic acid (●-----●) and the nucleic acids removed from *Staph. albus* (▲——▲) and *Cl. welchii* (○——○) by the action of lysozyme.

The preparations were hydrolysed by ribonuclease isolated from beef pancreas according to the method of Kunitz (1940).

The hydrolysis of the fractions produced by the enzyme at pH 6 was followed according to the method of Davidson & Waymouth (1944). Each tube of the experimental series contained 0·5 ml. of a solution of ribonuclease (1 mg.) in distilled water (5 ml.), 0·2 M acetate buffer pH 6·0 (1·5 ml.) and 2·0 ml. of a 0·1 % (w/v) solution of the bacterial nucleic acid dissolved in 0·001 M sodium carbonate and adjusted to pH 6 with 0·001 N hydrochloric acid (Fig. 2).

In view of the specificity of ribonuclease and, in particular, of the fact that it does not attack deoxyribonucleic acid, this result, coupled with the analytical data, establishes the identity of the bacterial fractions with ribonucleic acid.

The preparations could be recoupled with cells rendered Gram-negative by extraction with 5 % sodium cholate at 60° (method of Henry & Stacey, 1946). By this method the nucleic acid isolated from *Staph. albus* was 'replated' on to the Gram-negative cytoskeletons of *Cl. welchii* (Pl. 1, figs. 6, 7) and that from *Cl. welchii* on to the Gram-negative cytoskeletons of yeast (Pl. 1, fig. 10).

In preliminary experiments the nucleic acid was converted to the magnesium salt by heating it with an aqueous suspension of magnesium carbonate according to the original method of Henry & Stacey (1946). With the small samples of the bacterial nucleic acids available this procedure was not found satisfactory and alternative methods were investigated.

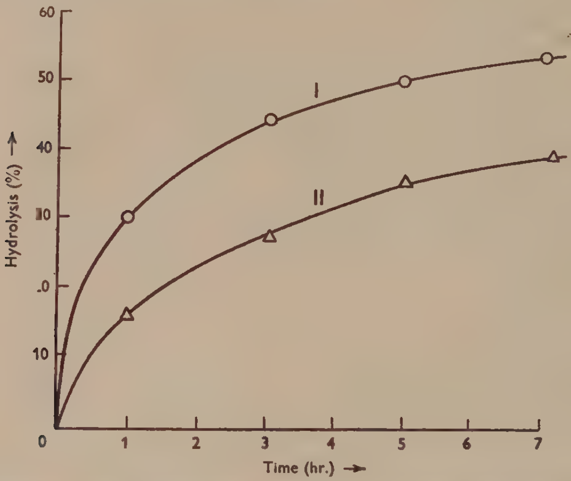

Fig. 2. Hydrolysis of the nucleic acids, removed from *Staph. albus* and *Cl. welchii* by lysozyme, with ribonuclease. △——△ nucleic acid from *Staph. albus*; ○——○ nucleic acid from *Cl. welchii*.

Since a solution of authentic magnesium ribonucleate gave ionic reactions for magnesium, it appeared possible that a solution of sodium ribonucleate in the presence of magnesium ion would function as efficiently as magnesium ribonucleate in recoupling experiments. It was found that the presence of sodium ion (0·5 M) had no inhibitory action on the combination of magnesium ribonucleate with the reduced cytoskeletons of *Cl. welchii*. Furthermore, as a result of quantitative experiments in which the reduced *Cl. welchii* cytoskeletons were added to serial dilutions of sodium ribonucleate in the presence of 0·012 M magnesium sulphate to give opacities corresponding to no. 10 on the standard barium sulphate opacity scale, it was established that as little as 0·1–0·2 mg. ribonucleic acid per ml. solution could thus be detected.

In contrast to the Gram-negative cytoskeletons obtained by sodium cholate extraction of Gram-positive organisms, killed cells rendered Gram-negative by the action of lysozyme and then reduced with formaldehyde could not be recombined with magnesium ribonucleate (Pl. 1, fig. 3). Since previous results had shown that lysozyme has no action on cells rendered Gram-negative with sodium cholate, cells rendered Gram-negative by lysozyme were subjected to extraction with the bile salt.

Extraction of cells rendered Gram-negative by the action of lysozyme with sodium cholate

Gram-negative staphylococcus cells remaining after the action of lysozyme (p. 267) were suspended in 5% (w/v) sodium cholate (30 ml.) at 60°. After 5 days the suspension was centrifuged and the clear supernatant (biuret positive) decanted. The residual cells were washed twice with distilled water and then suspended in 2% formol saline overnight. During this period the suspension agglutinated and separated as a fluocculent precipitate, although no disintegration of the cells occurred. After centrifuging, the cells were washed three times with distilled water and then resuspended in 4% magnesium ribonucleate. Stained smears prepared from the cells after 24 and 48 hr. at room temperature were, in each case, Gram-negative, showing that the cell bodies were not capable of recombining with ribonucleic acid.

Absolute ethanol (4 vols.) with 5 N acetic acid (1 ml.) was added to the sodium cholate extract and the resulting precipitate collected at the centrifuge after 18 hr. The solid was suspended in distilled water (20 ml.) and the suspension centrifuged after 20 min. at room temperature. The insoluble material (II) was again extracted with distilled water (10 ml.). The combined aqueous extracts (I) gave a positive Molisch reaction but a negative biuret. The insoluble fraction (II) gave a positive biuret and a negative Molisch reaction.

Addition of an equal volume of 0·25% (w/v) uranyl acetate in 2·5 (w/v) trichloroacetic acid resulted in the separation of a small precipitate which was collected after 6 hr. (supernatant, negative Molisch reaction). From this precipitate a fraction (2–3 mg.) was isolated, by the procedure previously outlined, which was identified as ribonucleic acid by the fact that it could be converted to the sodium salt and recombined with the Gram-negative cytoskeletons of *Cl. welchii* in the presence of magnesium ion, to restore the initial Gram-staining properties.

Fraction II, after drying with ethanol and ether, yielded 10 mg. of a light brown powder which was insoluble in water and dilute acids, but which was soluble in dilute alkalis. It gave a positive biuret, ninhydrin and Sakaguchi reaction, but negative Millon, sulphur and Molisch tests, and appeared to be an acidic protein or polypeptide.

DISCUSSION

Of the Gram-positive micro-organisms submitted to the action of lysozyme, only yeast proved resistant to the action of the enzyme. It would, therefore, appear that fundamental differences exist between the structures of the Gram complex of yeast and of bacteria. The experimental results have shown that when suitable killed Gram-positive organisms are rendered Gram-negative by the action of lysozyme, small, but definite, amounts of reducing substances are liberated into solution. A similar increase in reducing groups occurs when solutions of the specific polysaccharides of the Gram-positive organisms are incubated with lysozyme. These carbohydrates appear to be located at or near the cell surface since they may be isolated by procedures which are not accompanied by changes in the Gram-staining reaction or by disintegration of the cells. Furthermore, cells which have been rendered Gram-negative by extraction with sodium cholate—a process accompanied by the removal of ribonucleic acid and polysaccharide from the cell surface—do not liberate reducing substances when incubated with lysozyme.

Since killed Gram-positive organisms are completely resistant to the action of trypsin (provided the latter is free from ribonuclease activity), while cells which have been rendered Gram-negative by enzyme action or by extraction with sodium cholate are lysed, it would appear that protein material is not normally exposed at the cell surface. The outermost Gram-positive complex may, therefore, be considered to be composed of ribonucleic acid, specific carbohydrate and possibly lipid.

Concurrent with the partial hydrolysis of surface carbohydrate substances which occurs when lysozyme acts on killed cells, the Gram-staining reaction becomes negative and ribonucleic acid, the component of the complex responsible for the retention of the positive stain, is liberated into solution. This fact would suggest that, in the cell, ribonucleic acid and carbohydrate are in firm combination by means of linkages which are disrupted by the action of the enzyme. The isolation of a Gram-positive nucleoprotein (Henry *et al.* 1945) following a relatively short autolysis of *Cl. welchii*, together with similar findings for pneumococcal autolysis (Thompson & Dubos, 1938), shows conclusively that the ribonucleic acid of the Gram complex is also combined with cellular protein. Whether part of the latter together with ribonucleic acid is liberated from heat-killed organisms by the action of lysozyme remains to be determined. The evidence thus far obtained, however, leads to the conclusion that the major part of this protein is retained by the cell cytoskeleton.

Since all the killed Gram-positive organisms studied were, with the exception of *Sacch. cerevisiae*, susceptible to the action of lysozyme, while the specific polysaccharides of these organisms are known to be widely different in chemical structure, it is most probable that the action of lysozyme on the susceptible organisms is due to the ability of the enzyme to hydrolyse certain linkages common to these polysaccharides. Such linkages may well be those between carbohydrate and nucleic acid or between carbohydrate and protein. In support of this view is the finding that when bacterial cells or the specific polysaccharides from these cells are incubated with lysozyme the increase in free-reducing groups is relatively small. Meyer & Hahnel (1946) have, however, stated that lysozyme in low concentration presumably catalyses the depolymerization of its substrate with little or no apparent increase in reducing groups.

In contrast to the present results, Epstein & Chain (1940) were of the opinion that their polysaccharide substrate for lysozyme was contained within the bodies of the lysozyme-sensitive bacteria and they were unable to extract it with hypochlorite or formamide from the intact bacteria (*M. lysodeikticus*). However, as the authors were able to obtain complete lysis of suspensions of heat-killed *M. lysodeikticus* by the combined action of lysozyme and trypsin, it is apparent, in view of the foregoing discussion, that the enzyme also brought about the hydrolysis of some surface carbohydrate material with consequent disintegration of the Gram complex. Support for the present argument is provided by the recent work of Feiner, Meyer & Steinberg (1946) which has shown that the lysozyme substrate appears to be one of the antigens of *M. lysodeikticus*. It is, however, possible that two carbohydrates exist in *M. lysodeikticus*, both of which are susceptible to the action of lysozyme, the one forming part of the Gram complex and the other being contained in the somatic portion of the cell. The fortuitous occurrence of a carbohydrate substance in the cell bodies of *M. lysodeikticus* capable of being more completely hydrolysed by lysozyme than the carbohydrate of the Gram complex may in part explain the difference in susceptibility towards the enzyme which exists between *M. lysodeikticus* and most other organisms.

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EXPLANATION OF PLATE

The illustrations are prepared from direct contact prints taken from negatives on rapid process panchromatic plates. The bacterial cells, stained by Gram's method, are enlarged 1000 diameters.

Fig. 1. Heat-killed Gram-positive *Cl. welchii.*

Fig. 2. *Cl. welchii* cells rendered Gram-negative by the action of lysozyme.

Fig. 3. The Gram-negative cells shown in Fig. 2 treated with 2 % magnesium ribonucleate solution.

Fig. 4. *Cl. welchii* cells rendered Gram-negative by extraction with 2 % sodium cholate solution at 60°. (The variation in morphology apparent in this and the following photomicrographs of *Cl. welchii* occurred when other peptones were used in the culture medium in place of Evans's Bacteriological peptone and will be more fully discussed in a subsequent communication.)

Fig. 5. The Gram-negative cells shown in Fig. 4 recoupled with 2 % magnesium ribonucleate.

Figs. 6, 7. The Gram-negative cells shown in Fig. 4 recoupled with the magnesium salt of the nucleic acid removed from *Staph. albus* by the action of lysozyme.

Fig. 8. Cells of *Saccharomyces cerevisiae* after extraction with 2 % sodium cholate solution at 60°.

Fig. 9. The Gram-negative cells shown in Fig. 8 rendered receptive with formaldehyde and recoupled with 2 % magnesium ribonucleate.

Fig. 10. Cells shown in Fig. 8 rendered receptive with formaldehyde and recoupled with the magnesium salt of the nucleic acid removed from *Cl. welchii* by the action of lysozyme.

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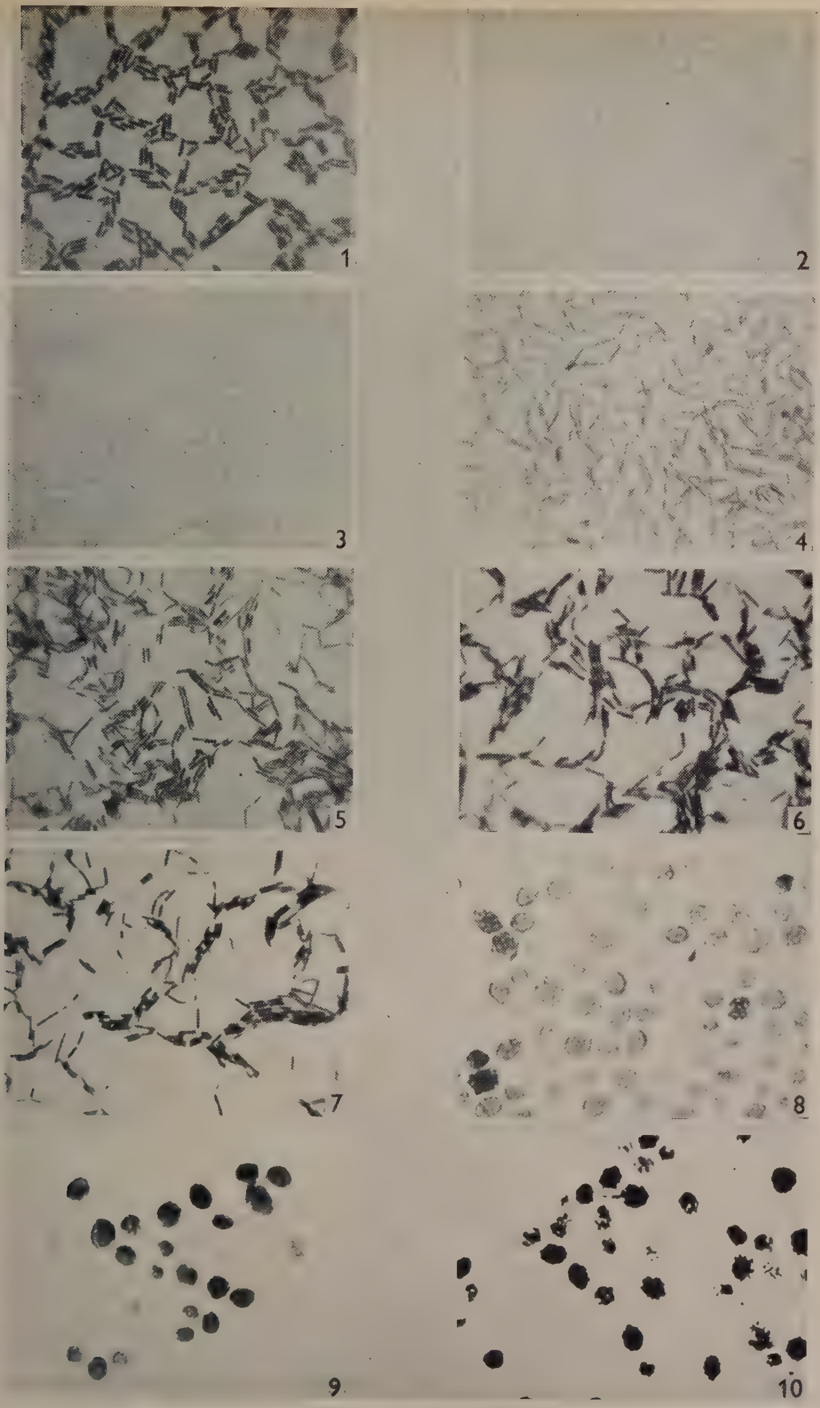

Figs. 1–10

M. WEBB—THE ACTION OF LYSOZYME ON HEAT-KILLED GRAM-POSITIVE MICRO-ORGANISMS.
PLATE 1

The Influence of Magnesium on Cell Division

1. The Growth of *Clostridium welchii* in Complex Media Deficient in Magnesium

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SUMMARY: The production of filamentous forms of *Clostridium welchii* which occurs in complex media containing certain commercial and chemically treated peptones is due to a deficiency of ionized magnesium. Such filaments revert to cells of normal morphological appearance on subculture in a medium containing free magnesium ions, but the change cannot be brought about by the presence of metallic ions other than magnesium. It is, therefore, suggested that the latter is essential for the activity of the cell-dividing mechanism.

The presence of a growth-inhibitory factor in certain peptones has been established. The active agent appears to be a fatty acid and may be extracted from acidified peptone solutions with ether or chloroform. The presence of the inhibitory substance in peptone markedly decreases the crop yield of *Cl. welchii* but has no direct influence on the production of filaments.

In the development of studies on the autolytic enzyme systems of Gram-positive micro-organisms (Jones, Stacey & Webb, 1948), it was observed that cultures of *Cl. welchii* in 2 % Difco Bacto-peptone broth differed considerably in morphological and autolytic characteristics from cultures in the normal growth medium which contained 2 % Evans's peptone.* In the former medium, *Cl. welchii* grew in the form of filaments of varying length (Pl. 1, fig. 1) which autolysed less readily than the short and more uniform ('normal') rods (Pl. 1, fig. 2) obtained by the cultivation of the bacillus in Evans's peptone. For example, the change in the Gram-staining reaction which occurs within 24–36 hr. when 'normal' cells are allowed to autolyse at pH 8·0 and 37°, occupied a period of 5 days when a suspension of the filamentous cells was allowed to autolyse under identical conditions. These long filaments reverted to cells of normal morphological appearance on subculture in Evans's peptone broth, indicating that the observed change was a direct response to some environmental stimulus.

On solid media, minute, circular, glistening colonies composed of long thin filaments (Pl. 1, fig. 3) were obtained when the organism was grown anaerobically on Difco peptone-agar plates, whereas cells from the larger colonies on Evans's peptone agar were smaller and more uniform in size (Pl. 1, fig. 4).

The use of other commercial peptones in the growth media for the cultivation of *Cl. welchii* yielded cells which varied in morphology from short rods (Difco Neopeptone), a mixture of short rods and filaments (Difco proteose peptone), to thin filamentous forms (Witte peptonum siccum). The organism failed to grow in a medium which contained 2 % Peptonum Siccum (Armour).

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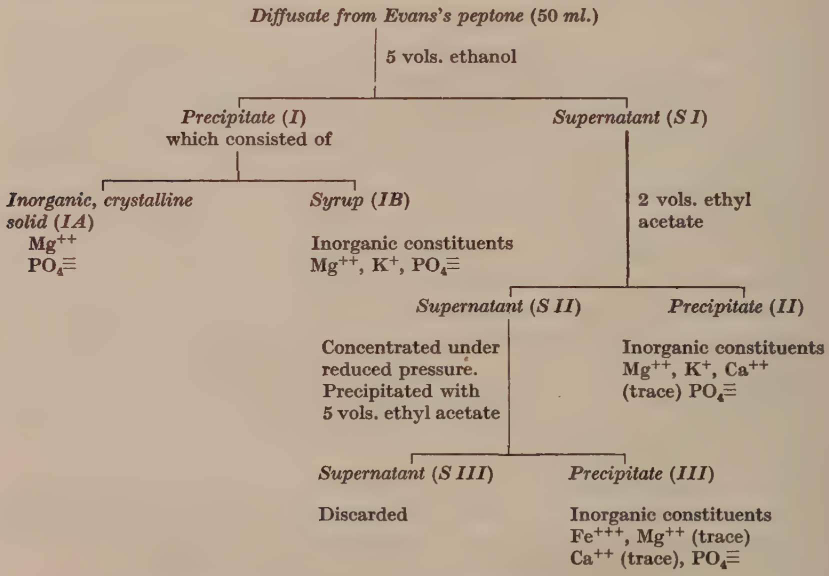
* Evans, Sons, Lescher and Webb, Runcorn.

From these results it was considered that the change in morphology associated with growth in the various media was due to differences in the chemical composition of the peptones, filaments being produced in media lacking or deficient in some component essential for normal cell division. Since normal growth and division occurred in media containing Evans's peptone, this was subject to fractionation as follows.

Dialysis of Evans's peptone. A solution of Evans's peptone (40 g.) in distilled water (250 ml.) was dialysed against distilled water (5 l.) for 24 hr. The diffusate was evaporated in an open vessel, finally on a water-bath, and the resulting syrup dissolved in distilled water (50 ml.). The dialysed peptone was diluted to a volume of 2 l. with distilled water containing sodium chloride (10 g.) and glucose (4 g.) and sterilized. The growth of *Cl. welchii* in this medium resulted in the production of filamentous cells which reverted to normal short rods on subculture in the medium containing, in addition, the diffusate at a concentration of 1 ml. in 40 ml.

Fractionation of the diffusate. Fractionation was carried out as shown in the following scheme. A sample of each precipitate was ignited in an open crucible and the inorganic constituent of the residual ash determined by qualitative chemical analysis.


Diffusate from Evans's peptone (50 ml.)
5 vols. ethanol
Precipitate (I) which consisted of
Supernatant (S I)
Inorganic, crystalline solid (IA)
Mg++
PO4≡
Syrup (IB)
Inorganic constituents
Mg++, K+, PO4≡
2 vols. ethyl acetate
Supernatant (S II)
Precipitate (II)
Concentrated under reduced pressure. Precipitated with 5 vols. ethyl acetate
Inorganic constituents
Mg++, K+, Ca++ (trace) PO4≡
Supernatant (S III)
Discarded
Precipitate (III)
Inorganic constituents
Fe+++, Mg++ (trace)
Ca++ (trace), PO4≡

Cl. welchii cultivated in the medium containing the dialysed peptone with the addition of a solution of I or II or of the inorganic constituents of I or II, yielded cells of normal morphological appearance. The reversion of the filamentous forms to cells of uniform normal size was also observed when the dialysed peptone was made up in saturated magnesium phosphate solution or in 0·1 % magnesium sulphate.

It was concluded that the production of filamentous forms of *Cl. welchii* in the dialysed peptone medium was due to magnesium deficiency. In the following section methods are described for the removal of magnesium ions from Evans's peptone. Although such methods are not specific for magnesium, the change in morphology associated with the growth of *Cl. welchii* in such media was reversed only by the addition of recrystallized magnesium salts. It was, therefore, concluded that other positive ions removed with magnesium from the peptone do not play any part in cell division.

Removal of magnesium ion from Evans's peptone

By 'ion-exchange' resin. A solution of Evans's peptone (5 g.) in distilled water (25 ml.) was passed through a column of Zeokarb H.I.P. (Permutit Co. Ltd., London), to remove free positive ions. The resulting acid solution (pH 4·9) was diluted to 250 ml. with distilled water containing sodium chloride (1·25 g.) and glucose (0·5 g.), adjusted to pH 6·2 and sterilized (Medium 47).

Precipitation with ammonium hydroxide. The presence of magnesium and phosphate ions in the diffusate from Evans's peptone suggested that the addition of ammonium hydroxide to a peptone solution would result in the precipitation of magnesium ammonium phosphate. It has previously been shown (Scudder, 1928) that organisms such as *B. alcaligenes* which produce alkalinity in peptone and meat infusion broth cause a rapid precipitation of magnesium ammonium phosphate in the medium.

A solution of Evans's peptone (10 g.) in distilled water (100 ml.) was made strongly alkaline with ammonium hydroxide (15 ml.) when precipitation occurred. After 18 hr. the solution was filtered and ammonia removed from the filtrate by aeration in the apparatus described by Davis & Daish (1913). The solution was concentrated in an open vessel on a boiling water-bath and made up for broth with the addition of 0·05 % K_2HPO_4 (Medium 23). Magnesium, calcium and phosphate were present in the precipitate.

Initially, 18 hr. cultures of *Cl. welchii* in Medium 47 were composed of short filamentous cells and many chains of short rods of normal size, but after two to three subcultures the organism grew as long filaments which were then invariably observed in all subsequent subcultures. Such filaments also resulted when the initial cultures in Medium 47 were incubated for 3 days. These observations indicate that the production of filaments is not due to the temporary alteration in enzyme balance brought about by adaptation of the organism to the new medium (cf. Spray & Lodge, 1943).

In Medium 23, filamentous forms (Pl. 1, fig. 5) were observed in the first and all subsequent subcultures of *Cl. welchii*. The cell deposit was gelatinous in character and aqueous extracts gave strong carbohydrate reactions. When the organism was subcultured every 24 hr. for several weeks in this medium and then subcultured into 2 % Evans's peptone broth the resulting growth was composed entirely of short ('normal') rods.

The effect of the reaction of the medium on the morphology of Clostridium welchii

Since the differences in the pH of the various media used (Table 1) may have contributed to the production of filaments, *Cl. welchii* was cultivated in 2 % Evans's peptone broth adjusted to various pH values by the addition of 0·1 N hydrochloric acid or sodium hydroxide. Buffers were not used, as these may produce specific effects (cf. Winslow & Falk, 1923). Control experiments showed that little change occurred in the pH of these solutions on sterilization. *Cl. welchii* failed to grow in all media of pH less than 5, normal cells were observed in cultures of pH 6·0–7·5, while in more alkaline media (pH 8·0–9·3) small oval cells, distorted curved forms and filaments were produced.

Table 1. pH *of nutrient media containing glucose* (0·2 %), NaCl (0·5 %) *and peptone* (2 %)

Peptone used in preparation of medium	pH of medium
Evans's	5·6
Difco Bacto	6·88
Dialyzed Evans's	5·0
'Ammonia-precipitated' Evans's	7·35

From these results it was concluded that the differences in pH of the media recorded in Table 1 had little, if any, influence in the production of the filamentous forms of *Cl. welchii.* The variation in morphology, as observed in cultures of pH 8–9, was not due entirely to the unfavourable pH, but to the precipitation of essential ions which occurred in these alkaline solutions. The evidence for this was as follows. Evans's peptone broth (300 ml.) was adjusted to pH 9·64 with N sodium hydroxide and heated at 100° for 30 min. when rapid precipitation occurred. After cooling, the suspension was stirred until homogeneous and 100 ml. removed. This fraction was adjusted to pH 6·2, when the precipitate redissolved, and sterilized (Medium 49). The residual alkaline solution was filtered, the filtrate adjusted to pH 6·1 and sterilized (Medium 50). Cells of normal morphological appearance were obtained when *Cl. welchii* was cultivated in Medium 49, but pleomorphism, with variation from normal rods to long filaments, was observed in cultures of the organism in Medium 50. Qualitative analysis of the precipitated solid revealed the presence of Mg^{++}, Fe^{+++} and $PO_4\equiv$.

The specific effect of magnesium

The change in morphology which occurred in media from which magnesium had been removed was not due to a low electrolyte concentration, since the growth of *Cl. welchii* in tubes of 'ammonia precipitated' Evans's peptone broth containing respectively 1·5, 2·5 and 3·5 % sodium chloride resulted, in each case, in the production of filamentous forms. Furthermore, determination of the relative osmotic pressures of the various culture media by means of membranes of copper ferrocyanide deposited on hardened filter-paper (Austin, Harntung & Willis, 1944), revealed only insignificant differences.

The addition of metals, such as calcium and copper, normally present in small amount in peptone and which would be coprecipitated with magnesium, to 'ammonia precipitated' Evans's peptone showed that such elements had no effect on cell division.

The addition of ferric iron (1·5 mg. $FeCl_3$) to 'ammonia precipitated' Evans's peptone (30 ml.) gave a medium which supported an exceedingly poor growth of *Cl. welchii* and some of the filamentous cells showed division into chains (Pl. 1, fig. 6). It was concluded that this latter effect was due to inhibition of the growth of the organism by the iron, the available magnesium present in the medium then being sufficient for division of a greater proportion of the cells.

Table 2. *Magnesium content of certain peptones*

Peptone	Water (%)*	Ash (%)	Magnesium (%) In ash	Magnesium (%) In peptone
Evans's	7·1	6·1	1·7	0·074
	7·3	5·9	1·8	0·077
Difco Bacto	3·6	3·6	1·8	0·048
	3·0	3·4	1·4	—
Dialysed Evans's	—	2·15	0·76	0·016

* Water (%) as loss in weight at 100°.

In support of this conclusion is the finding (see later) that, when *Cl. welchii* was grown in Difco peptone from which a growth-inhibitory substance had been removed, the resulting filaments were longer than are those which resulted when the bacillus was cultivated in normal Difco peptone broth which contained the growth-inhibitory substance.

Analysis of peptones

The magnesium content of the normal and chemically treated peptones was determined, as the evidence thus far obtained showed that the growth of *Cl. welchii* was influenced by the magnesium concentration of the medium and that inhibition of cell division occurred when this fell below a certain critical value. Samples of the peptones used were dried and ashed. The resulting inorganic residue was boiled for 30 min. with distilled water (100 ml.) containing concentrated sulphuric acid (2·5 ml.) and concentrated nitric acid (2·5 ml.) to convert pyrophosphate to metaphosphate. After cooling, the solution was filtered, the phosphate removed as ferric phosphate and the magnesium precipitated from alkaline solution with 8-hydroxyquinoline according to the method of Kolthoff & Sandell (1943). The washed precipitate was dissolved in dilute hydrochloric acid and, after the addition of a little more of the 8-hydroxyquinoline reagent, reprecipitated by the addition of ammonia. The final precipitate was collected in a sintered glass crucible, washed with dilute ammonia, dried at 110° and weighed. The results (Table 2) show that of the peptones examined, Evans's peptone had the highest magnesium content (0·075 %). Thus, in a medium containing 2 % Evans's peptone, the resulting concentration of magnesium ions (*c.* 0·0015 %) is such that normal cell division occurs throughout the entire growth of *Cl. welchii* that the medium is able to support. Since part of the magnesium present in the medium will be taken up by the organism to form the Gram complex (Henry & Stacey, 1946), it is concluded that the concentration of magnesium ions necessary for the activity of the cell-dividing mechanism is considerably less than this value. In 2 % dialysed Evan's peptone broth, which supports a heavy growth of *Cl. welchii*, the ionic magnesium concentration is exceedingly low (*c.* 0·00003 %) and is insufficient for both activation of the cell-division mechanism and the formation of the Gram complex. Consequently, in this medium, the organism grows as long filaments. The magnesium content of a 2 % Difco peptone medium is relatively high (*c.* 0·0009–0·0010 %), and it would be expected that this concentration would ensure the normal division of the poor growth that the medium supports. However, in contrast to Evans's peptone, the magnesium in Difco peptone appears to be firmly bound and little is present in an ionic form. That this is the case is shown by the following estimations of ionic magnesium.

Evans's peptone. Evans's peptone (10·0042 g.) in distilled water (100 ml.) was treated with ammonium hydroxide as before. After 30 min. the precipitate was collected in a sintered glass crucible, washed with dilute ammonia and dried at 110°. The solid (0·1024 g.) was dissolved in 5 N acetic acid (10 ml.) and the solution diluted to 250 ml. with distilled water. Quantitative determination of the ions detected by qualitative analysis, gave the following results: magnesium (precipitation with 8-hydroxyquinoline) 8·96 %; calcium (as $CaSO_4$) 8·37 %; ammonium (microKjeldahl) 2·7 %; P_2O_5 (by $PbMoO_4$ method) 55·0 %. The magnesium thus precipitated corresponds to 93·9 % of the total magnesium present in the peptone.

Difco peptone. The above procedure was repeated with Difco peptone. 9·9777 g. peptone yielded 0·0165 g. of precipitate which contained, in addition to some organic material, 29·3 % P_2O_5, but no magnesium precipitable with 8-hydroxyquinoline.

An increased amount of cell division was observed in cultures of *Cl. welchii* in 2 % Difco peptone broth which contained 0·1 % magnesium sulphate. The addition of magnesium ions to the culture medium, however, failed to increase the crop yield, and the cells retained their distorted appearance (Pl. 1, fig. 7).

The magnesium content of Clostridium welchii *when grown in Evans's and Difco peptone broth*

Cultures of the organism (18 hr. old) were centrifuged (Sharples supercentrifuge) and the cells washed with water (twice), precipitated with ethanol and dried with ethanol and ether. The dry cells were ashed and the inorganic residue analysed as previously described. The results (Table 3) revealed that the cells from the Evans's peptone medium contained more than twice the amount of magnesium contained by the cells from the Difco peptone medium. The results of Table 2 and 3 show that the growth of *Cl. welchii* in Evans's peptone medium utilized 11·1 % of the total magnesium present in the culture. The apparent anomaly that the magnesium removed from the Difco peptone medium by the growth of *Cl. welchii* only accounts for 2·5 % of the total available, may be explained on the assumption that only a small percentage of the magnesium of the peptone is in the ionic form. Such a conclusion would be in agreement with observations that magnesium is not precipitated from a solution of Difco peptone by the addition of ammonium hydroxide.

Table 3. *Magnesium content of* Clostridium welchii *grown in Evans's and Difco peptone media*

Peptone used	Yield dry cells/l. culture media (g.)	Ash of cells (Mean value) (%)	Magnesium (mean %) In ash	Magnesium (mean %) In cells
Evans's	0·5	7·3	5·4	0·35
Difco Bacto	0·15	6·5	2·5	0·16

Growth of Clostridium welchii *in 4% Difco peptone broth*

In experiments undertaken before it was established that much of the magnesium present in Difco peptone is not in the ionic form, *Cl. welchii* was inoculated into tubes of 4% Difco peptone broth under a liquid paraffin seal, as such a medium would have a magnesium content similar to that of 2% Evans's peptone broth (cf. Table 2). After 16 hr. at 37° the poor growth was subcultured into a second tube of the same medium. No growth ensued and the culture was apparently sterile, since subcultures in 2% Evans's peptone broth remained completely clear. Stained smears prepared from the first subculture in 4% Difco peptone broth showed short curved rods which, in contrast to cultures of similar age in the normal medium, were consistently Gram-negative. It was therefore concluded that there was present in Difco peptone a certain concentration of a bactericidal substance which in 2% Difco peptone broth caused partial inhibition, and in 4% Difco peptone broth complete inhibition of the growth of *Cl. welchii*. The observation that the cells from the first subculture in the 4% peptone medium were Gram-negative suggested that this substance was surface active (compare the use of surface active agents in the extraction of the Gram complex; Henry & Stacey, 1946) possibly a lipid or a fatty acid. Such a bactericidal substance was indeed isolated by extracting acidified solutions of the peptone with ether and other fat solvents.

Ether extraction of Difco peptone

A solution of Difco peptone (114 g.) in distilled water (500 ml.) was extracted three times with ether (which itself left no residue on evaporation). The combined extracts were dried ($CaCl_2$) and evaporated to give 0·01 g. of a yellow oil which was insoluble in water. The bactericidal substance was not removed by this procedure, for when *Cl. welchii* was inoculated into a medium containing 2% of this peptone the resulting growth was poor and was composed of distorted filamentous cells. The remaining aqueous solution was therefore adjusted to pH 3·5–4·0 with hydrochloric acid and re-extracted with ether (three times). Evaporation of the ether gave 0·14 g. of a brown, water-insoluble glass which was soluble in alkali and was precipitated as a brown oil on acidification of the alkaline solution. A solution of the sodium salt was 'salted out' on the addition of sodium chloride and gave a flocculent precipitate with calcium chloride. Fractions which possessed similar properties were also obtained by extracting the acidified peptone solution with chloroform.

The acid peptone solution after ether extraction was adjusted to pH 7·0 with N sodium hydroxide and then made up for broth in the usual way. Growth of *Cl. welchii* in this medium resulted in a greater crop yield (0·27 g./l.) compared with the yield in the unextracted Difco peptone broth (0·15 g./l., Table 3). These cells were filamentous in form (Pl. 1, fig. 8) and appeared longer than those from cultures in the unextracted medium (Pl. 1, fig. 1). Thus the presence of the antibacterial substance did not itself play any part in the production of the filaments. The cells contained 4·85 % ash which had a magnesium content of 3·62 %, corresponding to a concentration of this element of 0·17 % in the original cells. From the data of Table 2 it was calculated that, in this case, the magnesium removed from the ether-extracted medium by the growth of the organism amounts to 5·1 % of the total magnesium.

Cultures of *Cl. welchii* in 2 % ether-extracted Difco peptone media containing magnesium sulphate (50 mg./30 ml.) consisted of short ('normal') cells (Pl. 1, fig. 9), and a profuse growth of short, stout rods (Pl. 1, fig. 10) occurred in a medium which contained 4 % of the extracted peptone.

Cl. welchii failed to grow when inoculated into the 2 % ether-extracted peptone medium which contained a solution of the growth-inhibitory substance at a concentration corresponding to that present in the original unextracted peptone medium. As poor growth of the organism occured in a medium containing 2 % Difco peptone which contained the antibacterial substance, togéther with the fact that this substance cannot be extracted before acidification of the peptone solution, it is suggested that the fatty material is rendered less active by combination with other constituents of the peptone. In Evans's peptone broth containing 0·002 % (w/v) of the active substance the growth of *Cl. welchii* was poor and the cells were distorted in appearance; a concentration of 0·004 % (w/v) of the extract completely inhibited growth.

The bacteriostatic activity (serial dilution method) of the ether-soluble fraction against *Staphylococcus aureus* in Evans's peptone broth was 1:1000 after incubation at 37° for either 24 or 48 hr. It had no activity against the Gram-negative organism *Bact. lactis aerogenes.*

Extraction of other peptones

Acidified solutions of other commercial peptones were extracted with ether as above. The ethereal extracts were washed with water, dried ($MgSO_4$) and evaporated to dryness under reduced pressure. The properties of these fractions are recorded in Table 4.

The presence of an actively bacteriostatic substance in Peptonum Siccum (Armour) explains why *Cl. welchii* failed to grow in a medium containing this peptone. It would appear that the bacillus is exceptionally sensitive to these growth-inhibitory fatty acids, for both Peptonum Siccum and Difco peptone were used for the cultivation of strains of staphylococci, micrococci, and streptococci without any untoward effect.

The effect of subsequent addition of magnesium to young cultures of Clostridium welchii *in magnesium-deficient media*

The fact that when *Cl. welchii* is subcultured from a medium deficient in magnesium to one containing free magnesium ions the filamentous inoculum gives rise to a population in which normal cell division occurs, suggested the possibility of these ions causing the division of pre-existing filaments. In one series of experiments, sterile magnesium sulphate (1 ml. of 3 %) was added to 7 hr. cultures of *Cl. welchii* in 'ammonia precipitated' Evans's peptone broth and the cultures then incubated for a further 13 hr. At the end of this period, cells from control cultures containing no added magnesium were in the form of filaments and chains, whereas the growth in the experimental series contained filaments and shorter cells. Such observations show that if normal cell division occurs in a culture after the addition of magnesium at some time during the logarithmic growth phase, this addition does not cause the division of existing filaments. Furthermore, no detectable change in morphology occurred when filaments from an 11 hr. culture in the above medium were incubated with either 0·5 ml. 0·05 M magnesium sulphate or 0·5 ml. of an autolysed cell-free culture of *Cl. welchii* in Evans's peptone broth.

Table 4. *Comparison of the bacteriostatic activities of the ether-extractable substances from peptones*

Peptone	Ether extract yield g./100 g. peptone	Bacteriostatic activity against *Staph. aureus* after 48 hr. inhibitory dilution
Lab Lemco	0·135	1:500
Peptonum Siccum (Armour and Co.)	0·075	1:2000
Evans's	0·015	Inactive
British Drug Houses	0·030	Inactive

The relation of magnesium deficiency to the smooth-rough transformation

In some aspects the filamentous cells of cultures of *Cl. welchii* in magnesium-deficient media resemble the cells of rough variants of bacilli, for the smooth-rough transformation in some species is associated with recognizable changes in morphology (Wilson, 1930) and with an alteration in the method of cell division (Nutt, 1927). It has been reported (Henry & Stacey, 1943) that continued subculture of streptococci and certain other organisms in magnesium-deficient media gave rise to consistently rough colonies. However, in this case the smooth-rough transformation could not be induced in cultures of several strains of staphylococci and streptococci by continued subculture in 'ammonia precipitated' Evans's peptone media.

During the normal routine of subculturing the laboratory organisms, a rough variant of *Bact. lactis aerogenes* was isolated from a culture of the organism on Evans's peptone agar. It differed from the smooth variant in the following respects; in liquid media a fibrous pellicle was formed which showed a marked tendency to creep up the sides of the tube, the culture fluid remaining clear.

In contrast to the smooth form which grew throughout the liquid and gave rise to a considerable increase in viscosity, no polysaccharide was produced. On agar plates the variant grew rapidly, covering the surface with a spreading growth composed of irregular waxy colonies. The latter were completely removed from the agar on touching with the loop. In distilled water the growth proved difficult to emulsify, and in saline agglutination occurred. Stained smears and impression preparations showed the bacteria to be in chains.

Table 5. *Analysis of the rough and smooth forms of* Bacterium lactis aerogenes

Cells	Yield dry cells (g.) from 25 Roux bottles	Ash (%)	Magnesium (%)	
			In ash	In cells
Smooth organism	3·1	5·14–5·18	0·20	0·01
Rough organism	2·9	7·18–7·84	2·39	0·17

Twenty-five Roux bottles were inoculated with each variant and incubated at 37°. After 24 hr. the cells were removed in distilled water, washed twice at the centrifuge with distilled water and dried with ethanol and ether. Analysis of the two forms gave the results of Table 5 which show that the rough variant of *Bact. lactis aerogenes* contained a considerably greater percentage of magnesium than did the smooth form. It is, therefore, tentatively concluded that the inhibition of cell division which occurs in magnesium-deficient media bears no relation to the smooth-rough variation.

The similarity in morphology observed between the cells of cultures of rough bacilli and of cultures in magnesium-deficient media would suggest that the conclusion that an alkaline medium usually favours the $S \rightarrow R$ transformation (Hadley, 1927) should be accepted with reserve unless such observations are substantiated by immunological studies, since in alkaline media, magnesium may be precipitated as phosphate.

DISCUSSION

The production of filamentous forms of bacteria is well known and is observed when organisms are grown in the presence of sub-bacteriostatic amounts of certain antibacterial agents; as, for example, when *Bact. typhosum* is grown in the presence of methyl violet (Ainley-Walker & Murray, 1904), *Streptococcus viridans* in the presence of sulphonamide (Tunnicliff, 1939), *Cl. welchii* and other organisms in the presence of penicillin (Gardner, 1940) and *Bact. lactis aerogenes* in the presence of *m*-cresol (Spray & Lodge, 1943). It has also been claimed (Henrici, 1928) that filaments of *Escherichia coli* are produced when the surface tension of the medium is diminished by sodium ricinoleate to 35–40 dynes/cm. A similar effect has been observed in cultures of *Lactobacillus heleveticus* in which the surface tension of the medium has been lowered by the addition of sub-bacterio-static amounts of sodium glycocholate (Stacey & Webb, unpublished). In these latter cases it remains to be established whether the production of the long forms is a direct response to the lowering of surface tension or is due to the chemical nature of the surface active substances. Filaments have also been observed in bacterial cultures which have been subjected to the action of β- or γ-rays (Porter, 1946).

The conclusion reached from a survey of such observations is that the production of filaments in rapidly growing cultures is due to inhibition of cell division while growth, in the sense of synthesis of cellular substances, is unaffected. The diverse nature of the agencies which are able to produce such marked changes in morphology suggests that the enzymes of the cell-dividing mechanism are much less stable than are the enzymes concerned in synthetic processes. Lodge & Hinshelwood (1943) suggested that there are two independent factors, referred to as (L) and (D), which respectively control elongation and division during bacterial growth. It is assumed that (D) must be present in more than a critical concentration before division can occur. The enzyme systems responsible for the (L) and (D) factors are easily thrown out of balance and filaments are formed when elongation proceeds normally, but the concentration of (D) is below the critical. The rate of synthesis of the latter factor was thought to be decreased by the presence of antibacterial substances such as *m*-cresol (Spray & Lodge, 1943).

The present study has shown that cell division in cultures of *Cl. welchii* in complex peptone media is dependent on the presence of magnesium ions. In magnesium-deficient media cell division is inhibited and filamentous cells are produced. The observation that increased polysaccharide synthesis occurs in cultures of *Cl. welchii* in media from which the magnesium has been removed by precipitation with ammonium hydroxide may be explained by an alteration of enzyme balance, occurring when cell division is inhibited and giving rise to others.

Although magnesium appears essential for the growth of most bacterial species, and is almost invariably included in defined media, relatively little attention has been paid to its role in the growth and reproduction of bacterial cultures. That the element stimulates growth is evidenced by the work of Hotchkiss (1923), in which an increase in crop yield of *Esch. coli* in 1 % peptone was secured by the addition of magnesium chloride in low concentrations. Lodge & Hinshelwood (1939) found that a standard inoculum of *Bact. lactis aerogenes* failed to grow in a synthetic medium which contained less than two parts per million of magnesium. The conclusion of Olitzki & Bromberg (1931) that magnesium was completely unimportant for the growth of *Brucella* in complex media cannot be accepted, since peptones themselves contain relatively high percentages of magnesium (cf. Table 3). More pertinent to the present investigations are the observations of Palgen (quoted by Buchanan & Fulmer, 1930) that the average length of the cells in cultures of either *B. mesentericus* or *B. subtilis* is decreased by the addition of magnesium sulphate.

The nature of the enzyme or enzymes which constitute the cell-dividing mechanism is unknown. The process can apparently only occur in conjunction with the other stages of the growth and reproduction of bacterial cells, for the addition of magnesium or of an autolysed normal culture to existing filamentous cells does not result in division. Of great interest in this connexion is the discovery that deoxyribonuclease exists in pneumococcal autolysates (McCarty & Avery, 1946).

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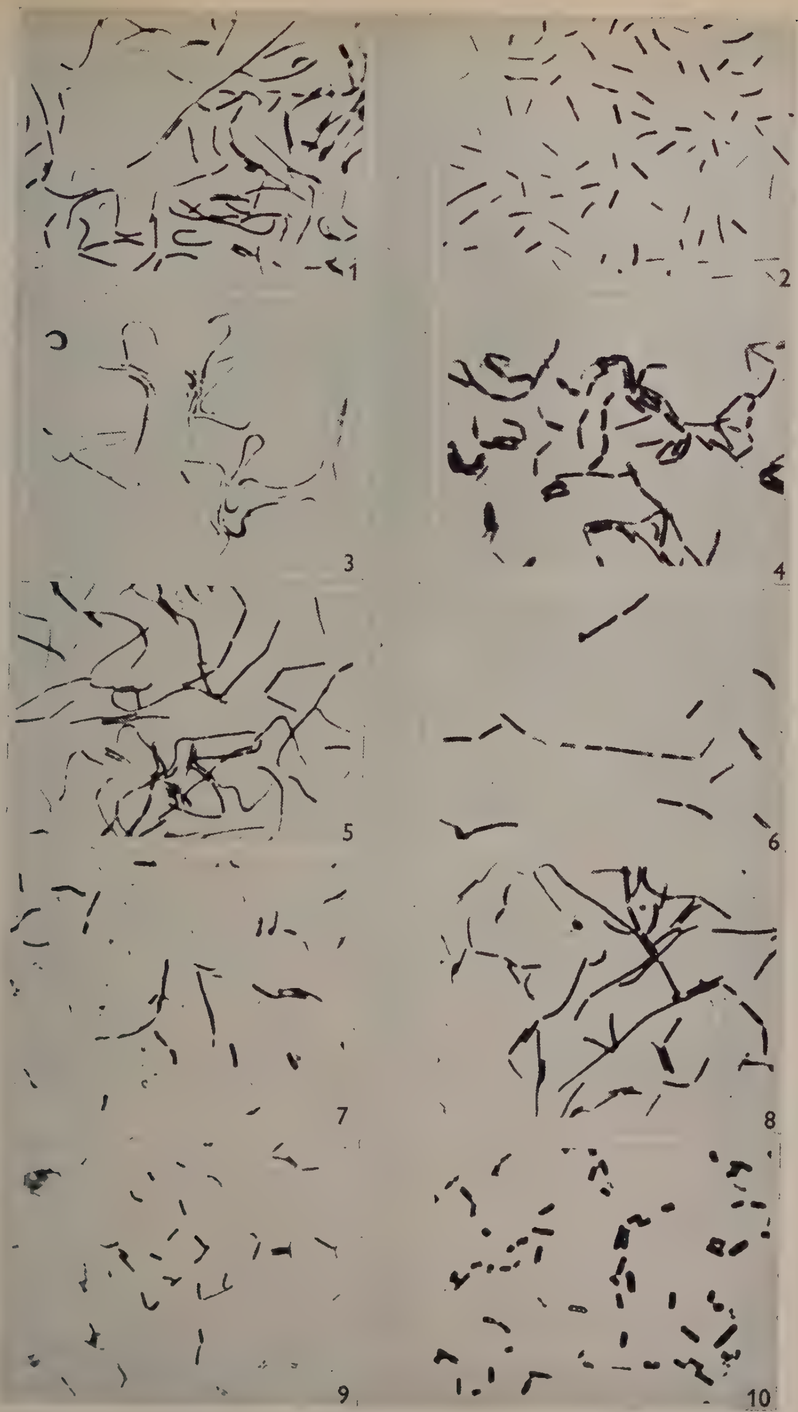
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Figs. 1–10

M. WEBB—GROWTH OF *CL. WELCHII* IN Mg-DEFICIENT MEDIA. PLATE 1

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EXPLANATION OF PLATE

Magnification, ×1200. Cells of *Cl. welchii* from cultures in:

Fig. 1. 2% Difco Bacto-peptone broth.

Fig. 2. 2% Evans's bacteriological peptone broth.

Fig. 3. Difco peptone-agar.

Fig. 4. Evans's peptone-agar.

Fig. 5. 2% Evans's peptone from which Mg^{++} had been removed by precipitation with ammonium hydroxide ('Ammonia precipitated Evans's peptone').

Fig. 6. 'Ammonia precipitated Evans's peptone' (30 ml.) + 1·5 mg. $FeCl_3$.

Fig. 7. 2% Difco peptone broth + 0·1% magnesium sulphate.

Fig. 8. 2% Difco Bacto peptone previously extracted with ether at pH 3·5 ('ether extracted Difco peptone').

Fig. 9. 2% 'Ether extracted Difco peptone' + 0·16% magnesium sulphate.

Fig. 10. 4% 'Ether extracted Difco peptone'.

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Preparation of Cell-free Bacterial Extracts with Powdered Alumina

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SUMMARY: A commercially available powdered alumina is very suitable for breaking bacterial cells according to the method of Wiggert, Silverman, Utter & Werkman (1940). Extracts have been prepared from streptococci in this way, which are capable of glycolysis, the deamination of adenosine diphosphate, and the production of NH_3 from arginine.

In using the method of Wiggert, Silverman, Utter & Werkman (1940) for preparing bacterial extracts by rubbing with powdered glass, the major operation at present is the preparation of the powdered glass, which is required in particles about the size of the bacterial cells. A preparation of alumina marketed as a fine abrasive for metallographic work was observed to consist of particles of about the size required, and has been found very suitable for breaking the cells of several bacterial species.

It was also found in at least one case that enzymically more active extracts were obtained by using the alumina than by using powdered glass. The powder added may well adsorb components from the cells which it breaks. The introduction of alumina for this purpose, therefore, has the advantage of introducing an agent which differs from glass in its adsorbing powers, as well as being easily available.

Whether the enzyme systems extracted were in true solution was not determined, but any particles with which they may have been associated were not resolved by a $\frac{1}{12}$ in. objective and hence were much smaller than the bacterial cells.

EXPERIMENTAL AND RESULTS

Alumina. Griffin and Tatlock's 'Microid Polishing Alumina', grade 3/50 ('slow cutting') was supplied as an aqueous suspension. It was centrifuged, the precipitate washed once with water, spread in the tube or on a dish and dried at 100°. This yielded a porous, friable solid, a little softer than blackboard chalk, which was not dusty and which could be handled without the precautions necessary in handling finely powdered glass. It was much more uniform in particle size than glass prepared according to Wiggert *et al.* (1940).

Breakage of cells. The procedure below is suitable for handling 0·4–3 g. moist weight of bacterial cells in 5–15 ml. centrifuge tubes, though the method is not likely to be limited to these quantities. Cells were collected by centrifuging in a tared tube, washed once, drained well from adhering liquid, weighed, and 2·5 times their weight of dried alumina added. The tube was cooled in ice-water, its contents mixed well with a glass rod to yield a dry powder, which was transferred to a cooled mortar. It was rubbed with a cooled pestle with maximum hand pressure for 30 sec., scraped together as it mounted the sides of the mortar, and the rubbing repeated. Indication of satisfactory breaking of the cells was given by the mixture darkening in colour and becoming moister. It was transferred (spatula) to a fresh, cooled centrifuge tube, the mortar and pestle washed with extracting solution, the washing added to the tube, and the whole mixed well and centrifuged. This yielded layers of the alumina below, a yellow juice above, and one or more layers of cell debris between. Separation of the grinding agent from the cell debris was much sharper with alumina than with glass powder, presumably because the alumina had been prepared in uniform particle size.

Degree of cell breakage. The fraction of cells broken by treatment was estimated by comparing the ratio of cells to alumina particles, before and after rubbing. Duplicate slides were made carrying in films of water (*a*) a specimen of the initial mixture and (*b*) a specimen after rubbing. These were dried, fixed, stained with methylene blue or by Gram's method and examined in cedar-wood oil or a substitute of similar refractive index. The alumina particles were then visible. In such preparations, glass particles are invisible, but similarly prepared slides with glass particles can be examined in water. The numbers of intact bacteria and of alumina particles in suitable fields of the four films were then counted. Reasonable agreement between the duplicates was found, provided the initial mixing had been thorough, fixing adequate, and blotting avoided in drying the slides. The bacteria apparently provided adequate anchoring material for the alumina particles. Gram-positive organisms were seen extremely sharply after partial breakage and Gram staining, against a background of unresolved (by $\frac{1}{12}$ in. objective) Gram-positive material and colourless alumina. Cells 'dented' or partly damaged by the alumina were only occasionally seen.

It is easy to break over 99 % of the cells of streptococcal preparations in this way. Breakage was most commonly less complete when the initial centrifuged mass of bacteria was too moist through inadequate centrifuging or draining from mother-liquor after centrifuging, though considerable latitude was permissible in these factors. Thus, preparations of moist weight/dry weight ratios between 6 and 9 were adequately broken when the ratios of alumina weight/moist bacterial weight were 2·5 to 3. With practice it is possible to add 2·5 times the bacterial weight of alumina, and to judge by the consistency of the mixture whether more alumina is likely to be needed: in the desirable consistency the mixture seems dry and can be moulded to shape, though it is more friable than would be desired for modelling It is, further, possible to tell by the 'feel' and appearance during rubbing with the pestle, whether breakage is occurring, and more alumina can if necessary be added at this stage Excess alumina rarely prevented breakage, but made the later extraction less complete.

Enzyme activity of cell extracts

Glycolysis in an extract from haemolytic streptococci. Cells of a β-haemolytic streptococcus (strain 'Richards', N.C.T.C. no. 5631), of wet weight 0·43 g. and wet weight/dry weight ratio 7·5, were broken with 1·1 g. alumina. The product was rubbed up with 0·85 ml. of a cold mixture of equal vol. of 0·05 M phosphates, pH 7·0 and a yeast extract (made by boiling baker's yeast with its own weight of water for 15 min., cooling and taking to pH 7). The mixture was immediately centrifuged in a tube packed in ice ('Bara' centrifuge, 5 min.). Warburg vessels had been prepared with reagents sufficient to give final concentrations of $NaHCO_3$, 0·03 M; glucose, 0·05 M; hexosediphosphate, 0·02 M. The extract (0·24 g., equivalent to 17 mg. dry weight of streptococci) was added, yellow phosphorus placed in a centre well, $N_2 + 5\ \%\ CO_2$ passed, the solution equilibrated at 37°, and readings commenced 7 min. after placing at 37° (40 min. after grinding the cells). CO_2 evolution was linear during the period of 7–80 min. at 37°, and at the rate of 14 μmol./hr. Chemical determinations (Friedemann & Graeser, 1933) showed over 85 % of this to be due to the formation of lactic acid. This rate of glycolysis represented about 10 % of the activity of the intact cells, and so compared favourably in activity with glycolyzing extracts from other bacterial cells. Extracts prepared from streptococci by ground glass were markedly less active. Other extracts prepared with alumina had 8–12 % of the activity in suspensions which contained less than half of the dry weight of the cells.

When glutamine (0·01 M) was added to a reaction mixture similar to that detailed above and containing a portion of the same streptococcal extract, additional NH_3 was found to be produced at the rate of 0·15 μmol./mg.-equiv. of juice/hr. during the glycolysis. This rate is also about one-tenth of that of the reaction in intact cells (cf. McIlwain, 1946).

Deamination of adenosine diphosphate. Streptococci (Richards's strain) were broken with alumina and extracted with twice their moist weight of 0·05 M phosphates, pH 7. With 0·02 M phosphates, pH 7, 0·03 M-$NaHCO_3$ and 0·05 M adenosine diphosphate in an atmosphere of 5 % CO_2 in N_2 (yellow phosphorus in a centre well), NH_3 was evolved at the rate of 0·25 μmol./mg.-equiv. of cells/hr. When adenosine diphosphate (0·05 M) was added to a glycolyzing streptococcal extract prepared and reacting under the conditions of the preceding section, additional NH_3 was formed at the rate of 0·5 μmol./mg.-equiv. of juice/hr.

Arginine dihydrolase. Preparations were made from streptococcal strains, by extracting 3 times with 0·6 % NaCl twice the moist volume of the cells. Reaction (cf. Hills, 1940) was carried out with the extract in 0·04 M phosphate, pH 7, containing arginine, 0·05 M. With the *R* strain, NH_3 was evolved at the rate of 0·5 μmol./mg.-equiv. dry wt./hr. This is about one-third of the total cell activity, but the pH optimum of the enzyme has not been fully investigated. It corresponded to 85 μmol./g. of liquid extract/hr. Activity was lost on heating at 100° for a few minutes.

Repeated use of powdered alumina

Under some circumstances the alumina may with advantage be used repeatedly. The following two methods were tried, using it for extracting arginine dihydrolase.

The deposit of alumina after grinding and extracting streptococci was washed once by centrifuging, suspended in 5 vol. conc. HNO_3, taken to dryness in a silica basin and then to red heat. Ignition left a light grey powder, still in particles of about the original size and shape. This was used for the breakage of streptococci, which followed its usual course: but the juice obtained on extraction was without arginine dihydrolase activity. The absorptive properties of the alumina with respect to the enzyme had presumably been changed; it was not further examined.

The deposit of alumina after grinding was washed twice with water, spread and dried at 100°, and used in the standard manner for extracting arginine dihydrolase from a second portion of the batch of streptococci used initially. The extract showed about 1·3 times the activity obtained from the first portion. Recovery was repeated and yielded again a juice of slightly higher activity. In this case cell constitutents from the earlier extractions presumably remained attached to the alumina and reduced the absorption in later extracts.

I am greatly indebted to Mr D. E. Hughes for assistance during this work, and to Mrs M. Bielschowsky for a gift of adenosine diphosphate.

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A Critical Study of Factors Influencing the Microbiological Assay of Nicotinic Acid

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SUMMARY: The cultural characters of a strain of *Lactobacillus arabinosus* and *L. helveticus* used in the microbiological assay of riboflavin and nicotinic acid proved to be typical, excepting that neither strain fermented xylose and the strain of *L. arabinosus* fermented rhamnose but not raffinose. Neither produced catalase, so that a strongly positive catalase test in these cultures indicates probable contamination by common air-borne micro-organisms.

Modifications of the medium to achieve maximum acid production were investigated by altering, omitting or adding various constituents. A modified medium was adopted allowing a slightly higher acid production. In this medium the maximum acid production was obtained in the presence of natural substances like peptone. The maximal acid production obtainable with nicotinic acid alone was slightly lower, suggesting an additional specific factor in peptone. No confirmatory evidence, however, could be obtained of its existence. Linoleic acid in a concentration of 640 μg./10 ml. medium depressed the acid production, and its action was antagonized by cholesterol. Other fatty acids and lipids had no effect.

A good reproducibility and a small coefficient of variation was found between tubes at various levels of nicotinic acid within any one assay, but between separate assays the variation was high (coefficient of variation = 12–19 %).

Since Snell & Wright (1941) published their original microbiological procedure for the estimation of nicotinic acid, it has been modified by a number of workers (Krehl, Strong & Elvehjem, 1943; Barton-Wright, 1944, 1945; Sarett, Pederson & Cheldelin, 1945). Experience in the assay of riboflavin with *Lactobacillus helveticus* showed that, by the use of an improved medium, a higher acid production and an increased reproducibility could be obtained (Kodicek, 1948; Pepper, 1947). An attempt was made to improve conditions likewise for the nicotinic acid assay, with *L. arabinosus* as the test organism.

EXPERIMENTAL

Cultural characteristics of Lactobacillus arabinosus *and* Lactobacillus helveticus

The characteristics and biochemical reactions of *L. arabinosus* were compared with those of *L. helveticus*. Snell's original strain *L. arabinosus*, 17–5 (Snell & Wright, 1941) was obtained from Dr Snell in 1944. After monthly subculture on yeast-water glucose agar slopes, and storage of the tubes at 4°, it was found in 1945 to be unsuitable for the assay of nicotinic acid. The bacteria had lost their sensitivity; their response to graded doses of nicotinic acid was very erratic, and the agreement between tubes at each level of addition was poor. A fresh culture kindly supplied by Dr Barton-Wright was investigated. It proved to be the *p*-aminobenzoic acid mutant described by Snell (see Shankman, 1943). Lewis also described this mutant strain (see Pennington, 1946) and stated that his stock culture, used as the test organism for this particular assay, suddenly acquired the ability to dispense with *p*-aminobenzoic acid.

The strain of *L. helveticus*, obtained from Dr Barton-Wright in 1943, was derived from Snell's original strain no. 7499 A.T.C. (Snell & Strong, 1939). It was in continuous use for experimental work from the time it was obtained and never showed any sign of mutation; its response to added riboflavin was constant and satisfactory.

Both strains had the following features, characteristic of members of the genus *Lactobacillus*: Gram-positive, non-motile, non-sporing, micro-aerophilic, aciduric, fermenting carbohydrates to give lactic acid but no gas, pleomorphism and irregular beaded staining in old culture, growing well only on complex media, indole negative, methyl red positive, and catalase negative. When testing the organisms for their sugar reactions, yeast extract was added to the tubes. When this was not done, falsely negative results occurred, merely because of lack of growth.

Wilson & Miles (1946) state that some of the Lactobacilleae are very slightly catalase-positive. With these two organisms there was no spontaneous evolution of gas upon adding H_2O_2, although in the course of several hours a few bubbles of O_2 did collect. The initial lack of reaction provided a quick and useful check test for contamination of the cultures. Since the common airborne bacteria are strongly positive, and contamination from bacteria other than these is very unlikely, an immediate production of O_2 is indicative of contamination.

L. helveticus ferments litmus milk rapidly, and after 48 hr. at 37° there is an acid clot and reduction in the lower portion of the tube; *L. arabinosus*, on the other hand, takes its place in Type B of White & Avery's classification (1910), producing insufficient acid to clot the milk (even after 5 days' incubation), although here again reduction occurs slowly in the lower part of the tube.

McIntosh, James & Lazarus-Barlow (1922) and Day & Gibbs (1928), working with lactobacilli associated with dental decay, reported that the reactions of individual strains were not constant. With *L. arabinosus* and *L. helveticus*, however, we obtained the same results (Table 1) on six occasions. Some of these fermentations were atypical according to Bergey (1939), but with one exception agreed with the findings of Camien, Dunn & Salle (1947), who studied extensively the effect of various carbohydrates upon the acid production of different strains of lactobacilleae; in this laboratory *L. arabinosus* has always fermented rhamnose but not raffinose.

Alterations of medium

The effect of altering single constituents of the medium for nicotinic acid assay was studied, in order to obtain an improved medium which would contain growth substances in optimal concentrations (see Leonian & Lilly, 1945); and allow for greater accuracy from a steeper response curve. Unless otherwise stated, Barton-Wright's procedure (1944) was used; the results reported are means of 3–6 tubes.

Modification of Barton-Wright's basal medium. The following alterations of Barton-Wright's basal medium (1944) were made. The quantities quoted refer to concentrations in 100 ml. of final basal medium.

(i) The glucose concentration was increased from 2 to 3 g./100 ml.

(ii) Potassium acetate was used instead of sodium acetate and the concentration increased from 2 to 3 g./100 ml.

(iii) Phosphate solution (20 g./100 ml.). The amount added was increased from 0·5 to 2 ml./100 ml.

Table 1. *Sugar fermentation tests*

Sugar	*L. helveticus*	*L. arabinosus*
Arabinose	−	+
Dextrin	−	−
Glucose	+	+
Dulcite	−	−
Galactose	+	+
Glycerol	−	−
Inosite	−	−
Inulin	−	−
Lactose	+	+
Fructose	+	+
Maltose	−	+
Mannitol	−	+
Mannose	+	+
Raffinose	−	−
Rhamnose	+	+
Salicin	+	+
Sorbitol	−	+
Sucrose	−	+
Trehalose	+	+
Xylose	−	−

(iv) A salt solution (*E*) containing Mg, Mn, Fe, Cu, Zn sulphates and NaCl (Table 6) was used in place of salt solution *B* (Snell & Strong, 1939).

These four modifications previously found optimal for the riboflavin assay (Kodicek, 1948) were adopted as being those most likely to encourage a high acid production by *L. arabinosus.*

(v) *Casein.* Spray-dried acid-hydrolyzed casein powder for microbiological assay work (Ashe Laboratories Ltd.) was tested in concentrations from 0·3 to 3 g./100 ml. As will be seen from Table 2, the blanks gave too high an acid response when concentrations of, or higher than, 1 g./100 ml. were used. The same maximum acid production was obtained both with 0·3 g. and with 0·5 g. casein, and 0·3 g./100 ml. was adopted for this particular sample of casein hydrolysate.

(vi) B *vitamins.* The concentrations of aneurin chloride hydrochloride, pyridoxin hydrochloride, *p*-aminobenzoic acid and calcium *d*-pantothenate were increased ten times from 0·01 mg./100 ml. to 0·1 mg./100 ml. each. The increased concentration of the B vitamins, however, had no significant effect on the acid production of *L. arabinosus.*

(vii) *Purines.* Adenine sulphate, guanine hydrochloride and uracil were added at different concentrations (Table 3). The concentration around

3 mg./100 ml. seemed to be about optimal and was adopted in the final medium.

(viii) *Cystine.* Increasing the concentration of L-cystine from 0·02 g. to 0·04 g./100 ml. produced no beneficial effect and, moreover, some of the cystine came out of solution when adjusting the pH of the medium to 6·8. The original concentration was therefore used throughout.

Table 2. *Effect of increasing the concentration of acid-hydrolyzed casein*

		Nicotinic acid (μg./10 ml. medium)				
	Concentration	0	0·05	0·1	0·3	0·5
Material	(g./100 ml. medium)	0·1 N-acid produced (ml.)				
Acid-hydrolyzed casein powder for microbiological work	0	0	1·3	1·7	2·8	3·0
	0·3	1·6	4·8	6·0	9·5	12·6
	0·5	1·7	3·4	5·1	9·0	11·5
	1·0	3·4	5·5	6·2	10·4	13·0
	2·0	5·5	7·6	8·6	12·2	14·1
	3·0	7·4	8·0	9·5	11·7	12·0

Table 3. *Effect of increasing the concentration of purines*

		Nicotinic acid (μg./10 ml. medium)				
	Concentration	0	0·1	0·2	0·5	1·0
Material	(g./100 ml. medium)	0·1 N-acid produced (ml.)				
Purine solution	0·001	0·7	4·0	6·2	10·3	12·1
	0·003	1·1	4·2	6·5	11·6	14·2
	0·005	0	3·4	5·8	11·3	11·5
	0·010	0	3·6	5·0	10·5	12·6

Purine solution contained adenine sulphate, guanine hydrochloride and uracil, 1 mg./ml. each.

(ix) *Buffers.* Various buffer solutions (Guirard, Snell & Williams, 1946) were tested instead of potassium acetate (Table 4). As they did not increase the acid production, 3 g./100 ml. of potassium acetate were retained.

Additions to the basal medium. The following sugars, which were fermented by *L. arabinosus*, were added in turn to the medium (already containing 3 g. glucose) in a concentration of 1·5 g./100 ml.: L-arabinose, galactose, mannose, mannitol, inositol and lactose. None of these compounds gave a significant increase in acid production. Acid production decreased when sucrose, but not when fructose, was substituted for glucose. Glucose was retained in the final medium.

Various other substances, including some of those reported by other workers as growth stimulants, were tested in turn. Under existing conditions no improvement was found on adding them in the following amounts per 100 ml. of final medium: Na pentose nucleotide 0·08 and 0·8 mg., dioxymaleic acid 6·4 mg., maleic acid 6·4 mg., succinic acid 6·4 mg., choline chloride 100 mg., folic acid 2·5 mg., asparagine 2000 mg., glutamine 0·1 mg., glutamic acid (added before and after autoclaving tubes) 0·1, 0·2 and 10 mg., methionine 1·2 mg. and thioglycollic acid 100 and 700 mg.

Table 4. *Effect of alterations of buffer*

Buffer	Added (g./100 ml. medium)	Nicotinic acid (µg./10 ml. medium) 0	0·5
		0·1 N acid produced (ml.)	
K acetate (own medium)	8	0	9·7
Na acetate	0·01	0·6	7·0
KH_2 citrate	0·25		
K acetate	0·01	0·5	7·4
KH_2 citrate	0·25		
Na acetate	0·1	1·5	9·3
KH_2 citrate	2·2		
K acetate	0·1	1·3	9·4
NaAc	2·2		

Table 5. *The effect of omitting individual constituents from the medium*

Constituents omitted	0·1 N acid production (at 0·5 µg. nicotinic acid level/10 ml. medium) (ml.)	% of acid production in 'complete' medium
0 (control 'complete' medium)	10·0	—
Glucose	0·4	4
Xylose	10·1	101
Potassium acetate	5·2	52
Casein, acid-hydrolyzed	0·7	7
Tryptophan	3·3	33
Cystine	6·7	67
Adenine sulphate	8·2	82
Guanine hydrochloride	8·3	83
Uracil	8·1	81
Xanthine	10·2	102
Biotin (free acid)	2·0	20
Aneurin	9·1	91
Riboflavin	10·1	101
Pyridoxin hydrochloride	8·6	86
Calcium *d*-pantothenate	5·3	53
p-Aminobenzoic acid	9·9	99
Salt solution A (phosphate)	5·8	58
Salt solution E	7·0	70
Ammonium sulphate	11·2	112

Omissions from the basal medium. The substances listed in Table 5 were omitted in turn from basal medium to which 5 µg./100 ml. of nicotinic acid had been added. The table gives the average results from three separate experiments. Since omission of xylose had no effect, and it was not fermented by *L. arabinosus*, it was not included in the final medium. Riboflavin was found to be non-essential, confirming the work of other authors (Snell & Strong, 1938; Krehl *et al.* 1943; Snell, 1945; Barton-Wright, 1945) and was also omitted, as were ammonium sulphate and xanthine. The maintained acid production of our strain of *L. arabinosus*, in absence of *p*-aminobenzoic acid, showed that we have the *p*-aminobenzoic acid mutant mentioned by Snell (Shankman, 1943; Shankman, Camien, Block, Merrifield & Dunn, 1947). The vitamin was, nevertheless, included in the final medium in case the mutant strain reverted to the normal type. Omission of the other constituents lowered the acid production, and they were retained in the final medium.

Table 6. *Table of composition of own final medium and of other authors*

Constituents*	Own medium (mg.)	Barton-Wright (1945) (mg.)	Krehl *et al.* (1943) (mg.)	Sarett *et al.* (1945) (mg.)	Woolley & Sebrell (1945) (mg.)
Glucose	3000	2000	2000	2000	2500
Potassium acetate	3000	2000 (NaAc)	2000 (NaAc)	1800 (NaAc)	2000 (NaAc)
Casein powder, acid-hydrolyzed vitamin-free	300	600	500	500	1000
L-Tryptophan	10	10 (DL)	10 (DL)	—	10 (DL)
L-Cystine	20	20	20	20	10
Adenine sulphate	3	1	1	—	1
Guanine hydrochloride	3	1	1	—	1
Uracil	3	1	1	—	1
Xanthine	—	1	—	—	—
KH_2PO_4	200	50	50	50	50
K_2HPO_4	200	50	50	50	50
$MgSO_4.7H_2O$	120	20	20	20	20
$MnSO_4.4H_2O$	3	1	1	1	1
NaCl	1	500	1	1	1
Xylose	—	100	—	—	—
Ammonium sulphate	—	300	—	—	—
Lloyd's reagent treated:					
Peptone	—	—	—	250	—
Liver extract	—	—	—	100	—
Yeast extract	—	—	—	100	—
Norite treated yeast extract	—	—	—	100	—
	(µg.)	(µg.)	(µg.)	(µg.)	(µg.)
$FeSO_4.7H_2O$	8	1000	1000	1000	1000
$CuSO_4.5H_2O$	8	—	—	—	—
$ZnSO_4.7H_2O$	8	—	—	—	—
Biotin (free acid)	0·04	0·04	0·02	—	0·1
Aneurin chloride hydrochloride	100	10	10	—	20
Pyridoxin hydrochloride	100	12	10	—	20
Calcium *d*-pantothenate	100	10	10	10	40
p-Aminobenzoic acid	100	10	10	—	20
Riboflavin	—	20	20	—	40

* Amounts stated per 100 ml. final medium (50 ml. of double-strength medium finally diluted to 100 ml.).

Results with the alterations in the medium

Table 6 shows the composition of our medium as finally modified and of the different basal media reported by other workers.

Our best response curve (Fig. 1) showed a greater acid production than that cited by Barton-Wright (1945), but taking the average response at different levels for 71 standard reference curves obtained in the course of over one year, the values for the improved medium were only slightly higher. The variation from experiment to experiment was considerable; for 71 experiments the coefficient of variation calculated for different levels of nicotinic acid ranged from 12 to 19 % (Table 7). On the other hand, in one single experiment the agreement between tubes at various levels of nicotinic acid was found to be very close and the variation small (Table 8).

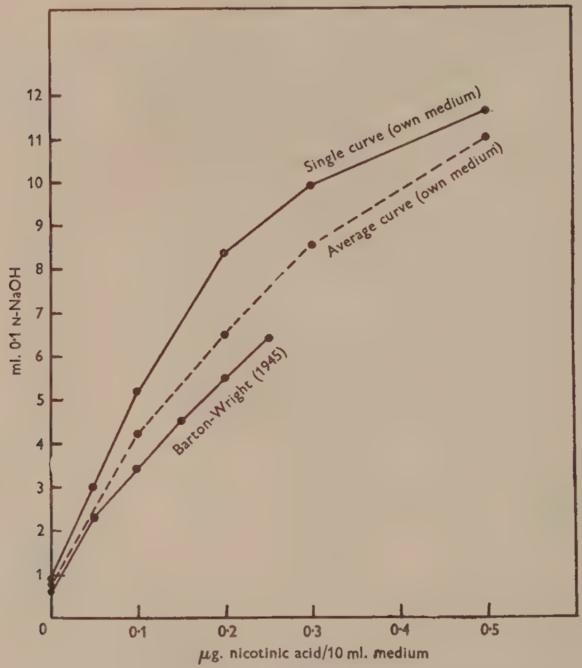


Fig. 1. Dose response curve of *L. arabinosus* in different media.

It was difficult to compare our average values for the standard reference curve with figures reported by other workers, as they do not indicate whether their curves referred to average values nor do they give any idea of their variation.

Because of the great variation in the acid productions from one experiment to another, the effect of altering the various constituents of the medium were tested within one experiment, and a control standard curve was always determined at the same time.

A similar erratic response was found when using *L. helveticus* with a certain riboflavin-free medium. When this was modified to give an acid production several times greater than the original, the results were then far more reproducible (coefficient of variation 3–7 %; Kodicek, 1948). Some deficiency of the medium may also be the reason for the erratic responses with *L. arabinosus*. As will be seen below, this organism is also capable of producing large amounts of acid of the order of that produced by *L. helveticus*.

Acid-producing potentialities of Lactobacillus arabinosus

In order to determine whether *L. arabinosus* was capable of such high acid production as *L. helveticus* in the riboflavin assay, *L. arabinosus* was seeded into a modified riboflavin-free medium containing 0·5 μg./10 ml. of added nicotinic acid. The acid production was between 22 and 27 ml. equivalents of 0·1 N-NaOH. When, however, peptone was omitted from the medium it no longer supported such high acid production. When photolyzed Bacto-peptone (Snell & Strong, 1939) was added in a comparable concentration, i.e. 100 mg./10 ml., to the modified medium used in the nicotinic acid assay, the acid production at 0·5 μg. nicotinic acid/10 ml. was increased from about 11 to 22 ml. equivalents. Untreated Bacto-peptone had a similar effect; in 13 experiments the average acid production was 21·3 ml. ± 0·698 equivalents. Similar high values, however, were also obtained when no nicotinic acid was added (Table 9).

Table 7. *Average acid production in modified medium and variation between experiments*

		Nicotinic acid (μg./10 ml. medium)							
	No. of exps.	0	0·05	0·1	0·15	0·2	0·25	0·3	0·5
Medium		0·1 N acid produced (ml.)							
Barton-Wright (1945)	—	0·9	2·3	3·4	4·5	5·5	6·4	—	—
Own medium	71	0·9	—	4·2	—	6·5	—	8·5	11·0
Standard deviation		±0·864		±0·802		±0·967		±1·025	±1·295
Coefficient of variation (%)		96		19		15		12	12

Table 8. *Variation between tubes*

Concentration of nicotinic acid (10 ml. medium)	No. of tubes	0·1 N acid produced (ml.)	Standard deviation	Coefficient of variation (%)
0·1 μg.	22	4·3	±0·258	6·0
0·2 μg.	22	7·1	±0·213	3·0

The nicotinic acid content of Bacto-peptone was found by chemical tests to be 37 μg./g. The addition of 100 mg. of peptone/10 ml. would add 3·7 μg. of nicotinic acid to the nicotinic acid-free basal medium. To decide whether the high acid production could be attributed solely to nicotinic acid, the vitamin was added in comparable amounts to the basal medium (Table 9). Addition of peptone resulted in a slightly higher acid production than that found upon addition of 5 μg. of nicotinic acid. When the '*t*' test was applied to test for the significance of the differences, the probability that these results were due to chance could not be excluded.

Further experiments were carried out to distinguish the peptone factor from nicotinic acid. The factor which seems to increase the acid production above that obtained with a corresponding level of nicotinic acid was stable to alkali, insensitive to light, not precipitated by 90% ethanol, not extractable by $CHCl_3$ at acid pH, adsorbed completely on activated charcoal at acid pH, and partially at neutral pH. These properties are similar to those of nicotinic acid and no conclusion could be drawn from these experiments. Further work on this matter is being carried out.

It is clear that *L. arabinosus* is capable of producing as much acid as *L. helveticus* (Fig. 2). The shape of the response curve is such that the increased acid production does not affect the lower portion of the response curve (between 0·1 and 0·2 μg. of nicotinic acid) which is used for assay work.

Table 9. *Comparison between acid productions in response to peptone and high amounts of nicotinic acid*

Addition per 10 ml. medium	No. of exps.	Average 0·1 N acid production (ml.)	Standard error of the mean	Remarks
Nicotinic acid:				
0·5 μg.	71	11·0	±0·154	—
5 μg.	14	18·6	±0·473	—
10 μg.	5	19·4	—	—
200 μg.	1	18·6	—	—
Bacto-peptone:				
100 mg.	9	20·1	±0·828	Equivalent to 3·7 μg. nic. acid
100 mg. + 0·5 μg. nic. acid	13	21·3	±0·698	Equivalent to 4·2 μg. nic. acid
100 mg. + 5 μg. nic. acid	2	27·8	—	—
100 mg. + 10 μg. nic. acid	1	23·5	—	—

'*t*' for difference of means between peptone and 5 μg. nicotinic acid = 1·76; '*t*' = 1·72 corresponds to $P = 0·10$.

'*t*' for difference of means between peptone with added nicotinic acid and 5 μg. nicotinic acid = 3·24; '*t*' = 2·79 corresponds to $P = 0·01$.

Inhibitory substances

Iron and calcium. In view of the findings of Chattaway, Happold & Sandford (1943), regarding the toxicity of certain cations, the effect of both iron and calcium was investigated in the modified final medium (Table 10) containing 0·1 and 0·5 μg. nicotinic acid/10 ml. respectively.

$FeSO_4.7H_2O$ was added to a final maximum concentration of 1 mg./100 ml. of medium. Calcium was added as $CaCl_2$ in concentrations of 10, 70 and 140 mg./100 ml. These amounts were chosen as being considerably in excess of concentrations likely to occur in extracts from most iron- and calcium-rich foods.

As will be seen from Table 10, there was no significant decrease in acid production in the presence of the iron salts or of $CaCl_2$. With extremely high concentrations of $CaCl_2$ (70 and 140 mg./100 ml.) there was a certain decrease in acid production only at the level of 0·5 μg. nicotinic acid.

Linoleic acid and other fatty substances. Unlike *L. helveticus*, *L. arabinosus* has been reported to be insensitive to fatty acids (Krehl *et al.* 1943). Their effect was tested on the organism used in this laboratory. Similar precautions were taken as in experiments with *L. helveticus* (Kodicek & Worden, 1945), and the tests were repeated with $CHCl_3$-extracted medium to prevent any interference

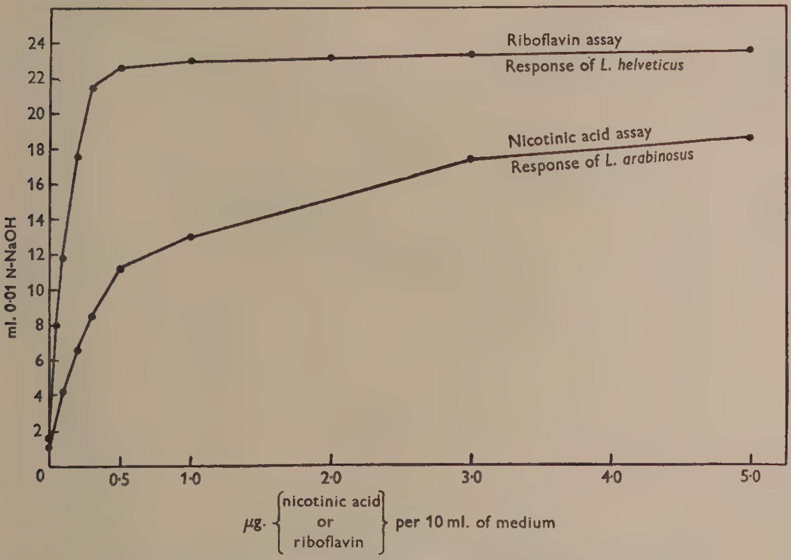


Fig. 2. **Maximum acid production of *L. arabinosus* and *L. helveticus* on addition of essential vitamin.**

Table 10. *Effect of increasing the concentration of iron and calcium salts*

Material	Added (mg./100 ml.)	Nicotinic acid (µg./10 ml.) 0·1	0·5
		0·1 N acid produced (ml.)	
Control	—	4·0	9·7
$FeSO_4·7H_2O$	0·1	—	10·9
	0·5	4·4	9·3
	0·10	4·5	9·8
$CaCl_2$ (dried)	10·0	—	9·9
	70·0	4·5	7·3
	140·0	5·3	7·7

from fat-soluble substances. The results are shown in Table 11. The acid production in $CHCl_3$-extracted medium was practically the same as that in the ordinary medium. There was almost complete inhibition of growth upon addition of 640 µg./10 ml. of potassium linoleate (prepared from pure methyl linoleate, iodine value 173). In presence of cholesterol (640 µg./10 ml.) this inhibition with linoleate did not take place. The inhibition by linoleic acid occurred, irrespective of whether $CHCl_3$-extracted or ordinary medium was used.

At a level of 160 μg. linoleic acid there was proportionally less inhibition. The depressant effect was more marked at higher levels of nicotinic acid (Fig. 3).

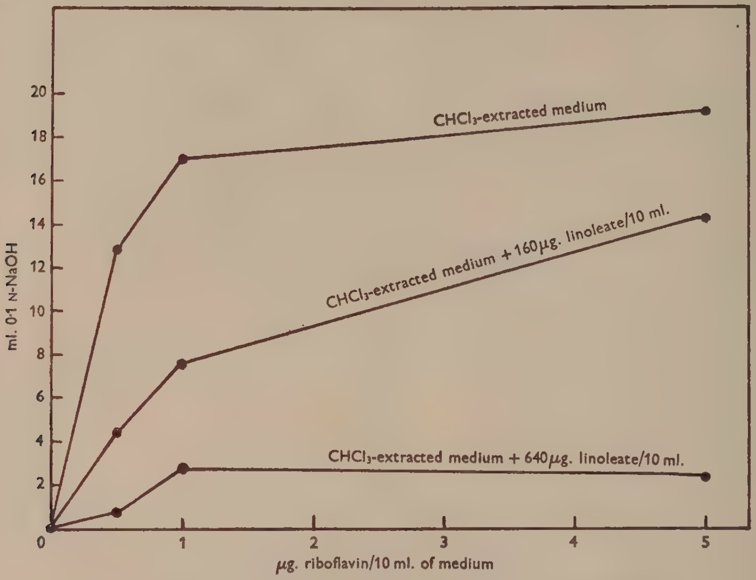


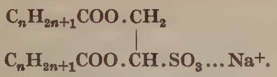
Fig. 3. Effect of linoleate on acid production of *L. arabinosus*.

Table 11. *Effect of fatty acids and lipoids*

Medium	Additions (640 μg./10 ml. medium)	Nicotinic acid (μg./10 ml.) 0	0·1	0·3	0·5	1·0
		0·1 N acid produced (ml.)				
Unextracted	—	0	4·0	8·7	11·5	13·1
$CHCl_3$-extracted	—	0	4·1	8·8	12·6	—
Unextracted	Linoleate	0	0	—	2·3	1·4
$CHCl_3$-extracted	Linoleate	0	1·1	—	0·5	2·0
$CHCl_3$-extracted	Linoleate and cholesterol	0	3·7	—	7·8	9·1
$CHCl_3$-extracted	Palmitic acid	2·0	4·7	—	12·0	—
$CHCl_3$-extracted	Stearic acid	0·4	3·8	—	10·2	—
$CHCl_3$-extracted	Lecithin	0·2	3·3	—	10·3	—
$CHCl_3$-extracted	Cholesterol	0·3	3·3	—	11·5	—

Palmitic and stearic acid, lecithin and cholesterol were each tested at a level of 640 μg./10 ml. (Table 11); none of these substances had a marked effect on the acid production.

Kodicek & Worden (1945, 1946) suggested that the inhibitory effect of linoleic acid might be due to the formation of a molecular monolayer on the surface of the bacteria. Accordingly, it was decided to investigate the effect of surface-active Aerosols. These compounds are long chain di-esters of sodium sulpho-succinate, e.g.



$$\begin{array}{l} C_nH_{2n+1}COO.CH_2 \\ \qquad\qquad\qquad\ | \\ C_nH_{2n+1}COO.CH.SO_3 \ldots Na^+. \end{array}$$

Two samples, Aerosol AY ($n=8$) and IB ($n=5$), were kindly supplied by the Colloid Science Department, Cambridge. As will be seen from Table 12, the Aerosols in concentrations of 640 μg./10 ml. showed no such inhibitory effect as was observed with linoleic acid. It seems that the effect of linoleic acid, if it is a physico-chemical one, is different from that of detergents like Aerosols.

Table 12. *Effect of linoleic acid and Aerosols added to* $CHCl_3$*-extracted medium*

	Nicotinic acid (μg./10 ml.)	
Addition (640 μg./10 ml. medium)	0	0·5
	0·1 N acid produced	
None	0·5	11·5
Linoleic acid	0	0
Aerosol 1B	0	12·3
Aerosol AY	1·7	11·5

DISCUSSION

The strain of *L. arabinosus* used in this laboratory proved to be the *p*-aminobenzoic acid mutant mentioned by American workers. Its cultural characteristics agreed well with published findings with the exception of its fermenting properties for raffinose and rhamnose. The former, contrary to findings of Camien *et al.* (1947), was fermented by the organism, while raffinose was not.

We can confirm the growth-promoting effect of peptone (Pollack & Lindner, 1943; Sarett *et al.* 1945; Camien *et al.* 1947), but we were unable to decide whether it was due to a specific factor or to the high nicotinic acid content of this substance.

We have omitted several constituents which were found to be non-essential for *L. arabinosus*, namely xylose, xanthine, ammonium sulphate and riboflavin, and, on the other hand, increased the concentration of glucose, potassium acetate, phosphates, magnesium, manganese salts and B-vitamins. Knight (1945) reported in his review that purines had definite growth-promoting effects on *L. arabinosus*. Sarett *et al.* (1945), however, found high amounts to be deleterious. We did not find this to be the case and increased the concentration of adenine, guanine and uracil. The amount of tryptophan was not altered, since no beneficial effect was found when increasing its concentration ten times above that used in the original medium.

Contrary to reports that iron salts exerted a marked depressing effect on certain bacteria, they did not interfere in our tests in concentrations which were in excess of those usually encountered when assaying iron-rich extracts. Calcium salts, only in a very high concentration, 70–140 mg./100 ml., decreased the acid production slightly, but such a high concentration of calcium is unlikely to occur in extracts of foodstuffs.

Linoleic acid inhibited the acid production of *L. arabinosus*. Higher concentrations were needed than those that were bacteriostatic for *L. helveticus*. This relative insensitivity may explain the negative findings of previous workers. The inhibition could be counteracted by cholesterol, as was the case with *L. helveticus*. The mode of action, if it is a physico-chemical one, would be different from that of such detergents as Aerosols which are ineffective in comparable concentrations. We can confirm that stearic and palmitic acids, cholesterol and lecithin have no enhancing effect on the acid production even at high concentrations. For assay work, the removal of lipids is advisable because of possible effects by unsaturated fatty acids, although the relative insensitivity of *L. arabinosus* makes this less essential than when working with *L. helveticus*.

The modified final medium fell short of our expectations of obtaining the high acid production experienced in assays with *L. helveticus*. Within any one experiment the results between tubes agreed closely, but a rather high variation between experiments was observed. This somewhat erratic response from one experiment to another will be referred to in another paper (Kodicek & Pepper, 1948) which deals with actual results on foodstuffs. There the reproducibility of the nicotinic acid values in repeats and the good agreement between chemical and microbiological values contrast pleasantly with our experience of the response of *L. arabinosus* towards nicotinic acid alone. Nevertheless, we feel that *L. arabinosus* is not the most appropriate organism for nicotinic acid assays. Its effective range for assay of nicotinic acid is far below its optimal requirements for this vitamin; under certain conditions it may, on the other hand, dispense with nicotinic acid (Shankman *et al.* 1947).

We wish to thank Drs A. E. Alexander and A. I. McMullen for kindly supplying us with samples of Aerosols. Our thanks are due to Dr L. J. Harris for his continued interest in this work and Dr C. G. McGaughey for the facilities kindly granted by the Institute of Animal Pathology, Cambridge.

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The Microbiological Estimation of Nicotinic Acid and Comparison with a Chemical Method

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SUMMARY: A modified basal medium was employed for the assay of nicotinic acid contained in various biological materials. Of the various possible comparisons of test material with standard nicotinic acid in the assay, the 'internal standard' method is preferable; by assaying the standard nicotinic acid in the presence of the test material, any discrepancies due to substances stimulating or inhibiting the nicotinic acid effect are minimized.

Of the different extraction procedures tested, treatment of samples with N-NaOH and N-H_2SO_4 gave the most satisfactory results; both N-NaOH and N-H_2SO_4 appeared to convert a 'precursor' in potato and bran, into nicotinic acid. 2N-HCl extracts yielded lower values, but the conversion of the precursor was less effective with potato than with bran, suggesting different precursors in the two vegetable substances.

The results of microbiological and chemical assay were in good agreement. The coefficient of variation of the microbiological results was of the order of 9 %.

In a previous paper (Kodicek & Pepper, 1948) we examined various factors which may influence the microbiological assay of nicotinic acid, and described a medium allowing a slightly higher acid production than with formerly described media. Within one experiment the agreement between tubes was close with a satisfactorily low standard deviation. Between different experiments, however, the variation for the respective levels was high.

This paper deals with the application of the method to extracts of natural foodstuffs, including methods of extraction and the computation of results.

MATERIALS AND METHODS

Nicotinic acid-free basal medium (double-strength):

Glucose (Analar)	6 g.	
Potassium acetate	6 g.	
Acid-hydrolyzed casein powder, spray-dried, vitamin-free (Ashe Labs. Ltd.)	0·6 g.	
L-Tryptophan	0·02 g.	(10 ml. of stock solution)
L-Cystine	0·04 g.	(10 ml. of stock solution)
Adenine sulphate	0·006 g.	(6 ml. of stock solution)
Guanine hydrochloride	0·006 g.	
Uracil	0·006 g.	
Biotin (free acid)	0·08 μg.	(0·8 ml. of stock solution)
Aneurin chloride hydrochloride	0·2 mg.	(0·2 ml. of stock solution)
Pyridoxin hydrochloride	0·2 mg.	(0·2 ml. of stock solution)
p-Aminobenzoic acid	0·2 mg.	
Calcium *d*-pantothenate	0·2 mg.	
Salt solution A	4 ml.	
Salt solution E	1 ml.	

The pH is adjusted to 6·8, using bromo-thymol blue as external indicator and the volume made up to 100 ml.; this is sufficient for 20 assay tubes. A suitable quantity of this medium is prepared the day before the experiment and stored overnight at 4°. It is advisable to check the pH immediately before the experiment.

Stock solutions. The stock solutions of L-tryptophan, L-cystine, adenine, guanine and uracil, biotin and salt solution A are prepared as described by Barton-Wright (1945). No toluene or other preservative is added, but all the solutions are stored in the refrigerator at 4°.

Aneurin solution. Dissolve 100 mg. of aneurin chloride hydrochloride in water, add five drops of conc. HCl and make up to 100 ml.

Pyridoxin, p-*aminobenzoic acid and pantothenate solution.* Dissolve 100 mg. each of pyridoxin hydrochloride, *p*-aminobenzoic acid and calcium *d*-pantothenate in water, add five drops of conc. HCl and make up to 100 ml. The solution is renewed every two weeks.

Salt solution E:

$MgSO_4.7H_2O$	60 g.	$FeSO_4.7H_2O$	0·004 g.
$MnSO_4.4H_2O$	1·5 g.	$CuSO_4.5H_2O$	0·004 g.
NaCl	0·5 g.	$ZnSO_4.7H_2O$	0·004 g.

Add three drops of conc. HCl and make up to 250 ml. with water. The solution keeps indefinitely.

Nicotinic acid standard. Prepare a solution containing 1 mg. nicotinic acid in 100 ml. of 20 % ethanol from a more concentrated solution of 100 mg./100 ml. Store in refrigerator and renew every three weeks. On the day of the experiment, it is convenient to prepare two weaker standard solutions for immediate use, containing 0·1 μg./ml. and 0·2 μg./ml.

Preparation of test extracts

Either of the two following methods is recommended (see also p. 310).

(1) N-NaOH *extraction.* Weigh into a beaker a representative sample of the foodstuff to be tested, containing about 30–40 μg. nicotinic acid, add 4 ml. 40 % NaOH and H_2O to 40 ml. Heat on a boiling water-bath for 45 min., agitating periodically. Cool, acidify to pH 4–5 and make up to 100 ml. with H_2O.

Centrifuge if necessary. Take 20 ml. or more of the digested extract and wash with an equal volume of $CHCl_3$ in a separating funnel. Wait until separation is complete. Discard the lower $CHCl_3$ layer, and centrifuge the watery layer to remove $CHCl_3$; final traces of $CHCl_3$ are evaporated off by heating on a water-bath, if necessary under vacuum. Take 10 ml. of the $CHCl_3$-washed extract, adjust to pH 6·8 with NaOH (bromo-thymol blue as external indicator) and make up to 100 ml. with H_2O.

(2) N-H_2SO_4 *extraction.* Weigh into a large conical flask a sample of foodstuff containing about 30–40 μg. nicotinic acid, add 8 ml. of 10 N-H_2SO_4 and water to a volume of 80 ml. Cover the flask with an inverted beaker, and autoclave at 15 lb. pressure for 1 hr. Cool, adjust pH to 4·5 and make up to 100 ml. with water. Centrifuge if necessary. Proceed with $CHCl_3$ washing as under (1).

Two levels of the final extract are tested microbiologically, 3 ml. and 5 ml. containing *approximately* 0·09–0·12 μg. and 0·15–0·2 μg. nicotinic acid, respectively.

Bacteriological procedure

Stock culture. Cultures of *Lactobacillus arabinosus* strain 17–5 are maintained on yeast water glucose agar slopes and subcultured at three-weekly intervals. After subculture they are incubated for two days at 37°, and then stored at 4°. Every four months they are tested for purity (see Kodicek & Pepper, 1948). A slope subcultured about twice weekly is used for preparation of the inoculum.

Standardization of the inoculum. The day before the experiment 20 ml. of the nicotinic acid-free medium are measured into a centrifuge tube (50 ml. capacity), 2 μg. of nicotinic acid added, and the volume made up to 40 ml. with distilled water. The tube is plugged and sterilized in the autoclave for 10 min. at 15 lb. pressure. When cool the medium is inoculated and incubated at 37° for 24 hr. It is then centrifuged, the supernatant medium decanted with sterile precautions and the bacterial deposit resuspended in about 50 ml. of 0·9 % sterile saline. The volume of saline usually need not be varied, but the concentration of bacteria per ml. is checked against Brown's opacity tube no. 1 (approximately 2×10^9/ml.). Originally the opacity was checked in a photometer, but the opacity-tube method is sufficiently accurate (the variation is ± 50 %) and more rapid. The experimental tubes are seeded with *c.* 0·04 ml. of standard inoculum.

The test is carried out as follows. Measure into bacteriological rimless (10 × 160 mm.) tubes 5 ml. of the double-strength medium.

Tubes for the standard nicotinic acid. Four tubes are used at each of the following levels of nicotinic acid: 0, 0·05, 0·1, 0·2, 0·3, 0·5 μg. per tube and the volume adjusted to 10 ml. with H_2O. As the standard nicotinic acid curve is not being used for the calculation of results, but only as a check for the linear dose-response, the number of tubes may be decreased.

Tubes for test extracts. Six tubes at each level of concentration of the final extracts to be tested are set up as follows:

	Double-strength medium (ml.)	Test extract (ml.)	Nicotinic acid standard added (0·03 μg./ml.) (ml.)	H_2O (ml.)
Level A	5	3	–	2
Level B	5	5	–	–
Level C*	5	3	1	1
Level D†	5	3	2	–

* (A + 0·3 μg. nicotinic acid.) † (A + 0·06 μg. nicotinic acid.)

Mix the contents of the tubes thoroughly and plug with non-absorbent bacteriological quality cotton-wool. The tubes are autoclaved for 10 min. at 15 lb. Using an upright type of autoclave, it is convenient to place the tubes in Petri-dish canisters; this prevents wetting of the plugs and darkening of the medium, probably because of residual air in the canister, which results in lower temperatures. Place in racks and inoculate as soon as they are cool. The tubes are incubated in their racks at 37° for three days. Daily shaking of the tubes does not appreciably affect acid production, as is the case with *L. helveticus*, and so is not practised.

Titration. It is most convenient to transfer the contents of each tube in turn to a small conical flask adding bromo-thymol blue as an internal indicator. The contents of the flask are titrated against standard 0·1 N-NaOH delivered from a self-filling burette. Visual comparison gives accurate readings without the use of a comparator-box. In the case of very dark or coloured extracts, the end-point may be doubtful and bromo-thymol blue is then used as an external indicator.

Computation of results. The standard nicotinic acid curve is plotted and values from extracts are compared to ensure that they lie on the linear part of the dose-response curve between 0·1 and 0·25 μg. nicotinic acid per tube. If these conditions are fulfilled, the calculation is carried out. An average is taken from all the tubes at each level. Using the response of 3 ml. of test extract as base, we compare the increased acid production due to a further 2 ml. of extract with that due to 0·03 and 0·06 μg. of nicotinic acid added to 3 ml. of test extract, respectively.

Let a = ml. of acid produced with 3 ml. extract,
b = ml. of acid produced with 5 ml. extract,
c = ml. of acid produced with 3 ml. extract + 0·03 μg. nicotinic acid,
d = ml. of acid produced with 3 ml. extract + 0·06 μg. nicotinic acid,

then

$$\textit{Nicotinic acid, } \mu g./g. \textit{ or ml. of test material} = 0{\cdot}25 \times \text{dilution factor} \times [0{\cdot}03\,(b-a)/(c-a) + 0{\cdot}06\,(b-a)/(d-a)].$$

EXPERIMENTAL

The extraction procedure

Snell & Wright (1941) and Krehl & Strong (1944) have shown that nicotinic acid compounds which are biologically active are also stimulatory for *L. arabinosus*. One exception is the precursor of nicotinic acid in cereals which *per se* has a negligible effect on this micro-organism (Snell & Wright, 1941; Krehl, Huerga, Elvehjem & Hart, 1946).

Kodicek (1940) noted that with cereals and cereal products the results obtained chemically varied according to the method of extraction and that alkaline treatment gave far greater values than water extraction or digestion with weak acids. This was attributed to the presence of a ‘chromogen’, the nature of which was uncertain. The findings were confirmed by Waisman & Elvehjem (1941), Oser, Melnick & Siegel (1941), Snell & Wright (1941) and Andrews, Boyd & Gortner (1942). Subsequently Krehl *et al.* (1946) showed that this ‘precursor’ possessed biological activity for dogs. Treatment with N-NaOH or N-H_2SO_4 converted it into free nicotinic acid which could be assayed microbiologically. Attempts to isolate this compound, however, were unsuccessful. The precursor was found in cereal products, especially in bran, and in potatoes.

Different chemical treatments were studied to find one which would extract all the nicotinic acid present in the materials and convert 'precursor' into microbiologically active substances. The five following methods were chosen as representative of possible chemical extraction procedures and were applied to various foodstuffs:

(*a*) N-NaOH extraction, water-bath 45 min. (see above).

(*b*) $N\text{-}H_2SO_4$ extraction, 15 lb. pressure, 1 hr. (see above).

(*c*) 0·05 N-NaOH extraction, 15 lb. pressure, 15 min.

Weigh out a sample of foodstuff (containing 30–40 μg. nicotinic acid), add 40 ml. of 0·05 N-NaOH; autoclave at 15 lb. pressure for 15 min. Cool and make up to 100 ml. Proceed as for extraction (*a*).

(*d*) 0·1 N-HCl extraction, water-bath for 40 min.

Heat the weighed material with 50 ml. of 0·1 N-HCl on water-bath at 100° for 20 min. Centrifuge and decant supernatant liquid. Resuspend residues in 30 ml. 0·1 N-HCl and extract for 20 min. on water-bath. Centrifuge and decant supernatant. Wash any remaining residues with 20 ml. 0·1 N-HCl, recentrifuge and add washings to combined supernatants; adjust to 100 ml. or note volume. Proceed as for extraction (*a*).

(*e*) 2 N-HCl digestion of 0·1 N-HCl extract, water-bath for 2 hr.

Take 50 ml. of extract (*d*) in an evaporating dish and add 14 ml. of conc. HCl (36 % sp.gr. 1·18) and heat for 2 hr. on boiling water-bath. Cool and adjust the volume to 50 ml. Proceed as for extraction (*a*).

Results

Table 1 shows the microbiological results obtained with various extraction procedures. The alkaline and $N\text{-}H_2SO_4$ extractions gave the highest values, especially with wheat grist, bran and dehydrated potato. This is in agreement with the findings of previous workers and is due to the presence of the precursor of nicotinic acid. Digestion with 2 N-HCl for 2 hr. did not yield as high values as the former extractions, but the results were higher than with a weak HCl extraction. The potato results are an exception; there the strong and weak HCl extraction gave identical values, a third of the total nicotinic acid content. Wheat flours of lower extraction gave values of the same order irrespective of the method of extraction. Foodstuffs with a low nicotinic acid content showed a greater variation between individual extraction procedures.

Improved results were obtained with egg-powder when this material was washed twice with equal volumes of light petroleum. The 2 N-HCl extraction gave a rather low value for fresh and dried milk, 0·7 and 7·0 μg. respectively, but from the few repeats we have done we were unable to decide whether this is a significant difference due to the extraction procedure. Fresh and dried milk, not extracted but merely diluted with distilled water, gave values of 1·0 and 7·5 μg. of nicotinic acid respectively.

The chemical results, so far as they were determined, were in good agreement with microbiological values. The extraction procedures using N-NaOH and N-H_2SO_4, giving the highest results and agreeing with chemical values, were adopted for the final method.

Table 1. *Bacteriological and chemical results from various extraction procedures*

	Nicotinic acid (µg./g.), using different extraction procedures									
	0·05 N-NaOH		N-NaOH		N-H_2SO_4		0·1 N-HCl		2 N-HCl	
Material	Bact.	Chem.	Bact.	Chem.	Bact.	Chem.	Bact.	Chem.	Bact.	Chem.
Wheat grist	66·1	52·8	71·7	59·6	79·8	74·9	55·0	—	48·0	52·5
Wheat flour, 85% extraction	20·7	—	22·9	—	18·3	24·5	19·2	—	20·6	18·8
Wheat flour, self-raising	21·0	—	23·7	—	20·9	23·2	20·2	—	25·4	25·6
Wheat germ	64·4	—	75·5	—	63·4	65·5	52·0	—	66·5	61·0
Wheat bran from 85% extraction flour	250	230	270	—	250	218	112	—	166	188
Potato, spray-dried	102	—	97·7	—	102	90·0	25·2	25·8	30·0	29·7
Potato starch	—	—	—	—	$<0·5$	$<1·2$	—	—	—	—
Milk, cows, fresh	1·1	—	1·2	—	—	—	1·0	0·9	0·7	0·7
Milk, cows, dried	8·5	—	8·8	—	—	—	9·5	9·4	7·0	6·8
Egg, spray-dried	2·6	—	4·8	—	4·3	—	5·2	—	3·1	—
Egg, spray-dried washed with light petroleum	—	—	—	—	3·2	3·9	—	—	2·6	3·7

Methods of computation compared

The response in acid production of *L. arabinosus* to additions of nicotinic acid is amenable to different methods of calculation. The original method proposed by Snell & Strong (1939) and adopted by early workers in this field was to plot a standard reference curve from graded doses of the vitamin and to read off and calculate from this the content of the test materials.

This method has been criticized by Wood (1946) and both he and Finney recently have devoted much attention to the design and statistical analysis of microbiological assays and computation of results (Wood & Finney, 1946).

Wood proposed two methods of computation, as an improvement of the 'direct reading method'. The premises on which these methods are based, are '(*a*) that the response supposed to be produced by the known amounts of factor *X* is actually due to the factor itself; and (*b*) that the response is also due solely to the presence in it of factor *X* without augmentation, diminution, or modification by any other substance also present.... If in any particular assay this hypothesis is not valid, then the results obtained will clearly be inaccurate' (Wood, 1946). We agree with Wood that the bio-assayist who is determining the amount of some vitamin contained in a foodstuff can never be sure, merely because all previous assays have been satisfactory, that the present one will be, and we considered that assumption (*b*) was not necessarily valid for all tests materials.

We have therefore applied another method of calculation which may to a certain extent correct for any stimulation or inhibition, i.e. the internal standard method. The microbiological assays were performed in such a way as to allow the calculation by four methods; namely, direct reading (Snell & Strong, 1939); slope ratio (Wood, 1945); common zero five-point assay (Wood, 1946); and the internal standard method. In this last method the calculation is based on acid production of test extracts and nicotinic acid added to the test extract. The standard reference curve is used to ensure that the levels tested are within the steep linear portion of the dose-response curve, thus establishing the fact of maintained acid production and the straight-line relationship.

Table 2. *Effect of extracts on the response to additions of nicotinic acid*

	Standard reference curve (0·1 µg. nicotinic acid)		Extracts (3 ml.)			
			Cocoa		Maize	
Additions	Acid (ml.)	Increase	Acid (ml.)	Increase	Acid (ml.)	Increase
—	3·9	—	3·5	—	4·2	—
Nicotinic acid (0·03 µg.)	4·8	+0·9	4·4	+0·9	5·4	+1·2
Nicotinic acid (0·06 µg.)	5·6	+1·7	5·2	+1·7	6·6	+2·4

The results were computed for thirteen samples by the various methods and compared with those of chemical estimation. The difference between the results calculated by the various methods was not statistically significant. Nevertheless, we finally adopted the 'internal standard' method, because in each test any unspecific stimulatory or inhibiting substances present might be expected to affect to the same degree the response of the bacteria both to any nicotinic acid present originally in the extract and to the nicotinic acid added to the extract. Table 2 shows the results of an assay of extracts of cocoa and maize. In the standard reference curve additions of 0·03 and 0·06 µg. nicotinic acid above the 0·1 µg. level gave a response of 0·9 and 1·7 ml. of acid, respectively. The same additions to 3 ml. of cocoa extract gave the same response, but to 3 ml. of maize extract, the response found was 1·2 and 2·4 ml. This suggests that calculations from the standard reference curve may be misleading, and that our method does in fact allow, at least to some degree, for unspecific effects by substances in the extracts.

Comparison of microbiological and chemical estimates; reproducibility of results

In addition to the comparisons listed in Table 1, the method finally adopted was used for assays on various other materials and the values obtained compared with chemical results (Table 3). The agreement was good. The reproducibility of results was tested by repeated assays on yeast and bran (Table 4). The coefficient of variation was about 9 %. The coefficient of variation for pure nicotinic acid was between 12 and 19 % (Kodicek & Pepper, 1948).

Table 3. *Further comparisons of microbiological and chemical results*

Material	Nicotinic acid (μg./g.) Microbiological method	Nicotinic acid (μg./g.) Chemical method (Wang & Kodicek, 1943)
Kipper (dehydrated)	142·0	138·0
Liquid liver extract	123·0	120·0
Herring (dehydrated)	81·0	99·3
Malt extract, liquid	74·3	68·0
Whale meat	49·3	56·2
Muscle (beef)	36·7	36·7
Pea meal	25·6	23·3
Semolina	17·1	19·2
Maize meal	15·0	15·5
Cocoa	17·2	16·1
Oats (ground)	6·7	6·2
Carrot (fresh)	6·3	4·3
Tomatoes (fresh)	5·1	5·9
Plums (fresh)	4·1	4·2
Raspberries (fresh)	3·7	2·4
Blackberries	1·7	1·3
Urine (rat)	0·9	1·2

Table 4. *Reproducibility of microbiological results*

	No. of samples	Nicotinic acid (μg./g.)	Standard deviation	Coefficient of variation (%)
Yeast	5	348·8	±30·9	8·9
Bran	5	250·2	±23·7	9·5

DISCUSSION

The extraction of foodstuffs with either N-NaOH or N-H_2SO_4 was found to be the most suitable. Both extraction procedures gave similar values even in foodstuffs known to contain the precursor of nicotinic acid. These findings could be confirmed by chemical assays. It is interesting that 2 N-HCl digests of bran gave a nicotinic acid value only 66 % of that obtained in NaOH and in H_2SO_4 extracts. The 2 N-HCl extraction of potato powder yielded an even lower proportion of the total nicotinic acid content; and extraction with 0·1 N-HCl gave low values for both bran and potato powder. With potato the values were of the same order as those of the 2 N-HCl extraction, suggesting that 2 N-HCl treatment did not convert the 'precursor' as it did in bran. We have no explanation for this finding; possibly the precursor in potatoes is different from that in bran. If the biological activity possessed by the precursor extends to man, then the potato takes its place as an important source of nicotinic acid for, on the assumption of a daily consumption of 200 g. fresh potato, it would provide nearly one half of a man's daily requirements.

The microbiological results were in good agreement with chemical results. The variation between replicates, however, was high (9% coefficient of variation), and we feel that there is scope for further improvements in the microbiological method. The results by various computation methods were not significantly different. The 'five-point zero' assay and the 'internal standard' method are both amenable to statistical analysis and will detect non-valid assays. We prefer to use the internal standard method because it may reduce the error due to the effect of substances other than nicotinic acid present in the extract, and because of its simplicity of calculation. The computation by the internal standard method is only valid if the addition of nicotinic acid to extracts does not increase the acid production above the workable linear range, and this we can check by the use of the standard reference curve.

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The Mechanical Destruction of Bacteria

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SUMMARY: A number of different organisms were subjected to violent shaking with minute round glass particles. Vegetative bacteria, spores, and acid-fast species were killed by this treatment, though at varying rates.

Curran & Evans (1942) described the destruction of bacterial spores by violent agitation with small inert particles. Several workers (e.g. Gale, 1947) have applied this technique to the extraction of enzymes from vegetative cells. Unlike other mechanical methods (e.g. sonic disintegration or the use of bacterial mills) it requires no elaborate equipment, and the risk of damage to proteins or other labile cell constituents is less than with methods involving autolysis or the use of chemical agents. Curran & Evans (1942) obtained large decreases in the viable count of spore suspensions by this method and claimed (though without giving experimental data) that suspensions of *Bacterium coli* may be completely sterilized. We have investigated a wider range of both sporing and non-sporing bacteria. In confirmation of Curran & Evans's findings we obtained a rapid exponential fall in the number of viable organisms: but a small proportion of the cells appeared to be much more resistant to this treatment than the others present in the same bacterial suspension.

METHODS

Bacterial suspensions. The organisms tested were mainly from our laboratory stock. They were grown for 24 hr. (48 hr. for *Mycobacterium smegmatis*) at 37° on the surface of nutrient agar, with addition of 0·1 % glucose and 10 % horse blood for the pneumococcus, washed off with physiological saline, washed twice with saline, and resuspended in the fluid (usually saline) in which they were to be shaken. The cultures of *Bacillus subtilis*, *B. mesentericus* and *B. anthracis* were allowed to stand at room temperature for a few days before preparing the suspensions, which consequently consisted mainly if not entirely of spores. Suspensions of yeast were prepared from commercial brewers' yeast by repeated washing with saline.

All suspensions were adjusted to an opacity equal to Brown's tube no. 3, which corresponds to about 10^9 organisms/ml. in most cases. Exact adjustment of the initial count was not practicable since, for reasons stated later, the 'initial' was taken after a short period of shaking during which a variable degree of killing took place. All precautions to avoid extraneous contamination were taken throughout.

Particles. Curran & Evans (1942) employed several types of particles and found small smooth glass beads to be the most suitable. We obtained these from Messrs Chance Bros. Ltd., Smethwick, England, under the trade name 'ballotini'. Three grades were supplied whose mean diameters, measured under the microscope, were: grade 6, 0·80 mm., grade 9, 0·26 mm., grade 12, 0·13 mm. Grade 9 was used unless otherwise stated; grade 12 may be more efficient, but supplies were limited at the time.

Shaking machine. An ordinary laboratory shaker was used, accommodating two 4 oz. medicine type flat bottles laid lengthwise on their narrow sides. Speed was 300–350 strokes/min. with an amplitude of 2½ in. There was no means of ensuring that speed remained constant, and the electric main supply was subject to serious fluctuations. The agreement between replicates indicated, however, that variations in the speed of the shaker did not materially affect our results.

Procedure. The beads were washed with chromic-sulphuric mixture, rinsed thoroughly with water followed by acetone, dried and sterilized in the hot-air oven before each experiment. 40 ml. of bacterial suspension, prepared as described above, were placed in one sterile 4 oz. bottle with 50 g. of beads. The bottle was closed with a rubbler-lined screw cap and placed in the shaker. After 15–20 min. shaking a 1 ml. sample was aseptically withdrawn and a viable count made. This was taken as the 'initial' count. A count taken before shaking was begun was liable to give misleading results as the organisms, in some cases, adhered together in clumps. Further counts were made at intervals of 1–2 hr. up to 10 hr., and a final count, in some experiments, after 24 hr. shaking. The experiments were performed at room temperature, usually about 10°. The prolonged shaking did not produce any appreciable rise in temperature but when speed was increased to 600 strokes/min. a rise of a few degrees was observed.

Viable counts. 1 ml. of the suspension was withdrawn and serial tenfold dilutions in saline prepared. 1 ml. of each of four suitable dilutions were placed in Petri dishes and 15 ml. melted nutrient agar added; for the enterococcus and the pneumococcus this medium was enriched with 10 % of serum. Colonies were counted after incubation at 37° for 48 hr. (4 days for *Myco. smegmatis*) and the number of viable organisms/ml. estimated. When spore counts were required, the vegetative forms were killed by heating for 15 min. at 70°. With yeast, the counts were made by stroking one loopful (0·001 ml.) on the surface of a Petri dish containing Sabouraud's medium and incubating at 20° for 3 days.

RESULTS

Method of expressing results. Since the killing of micro-organisms by this method (in common with most sterilization processes) followed an exponential law, results were expressed by plotting the logarithm of the fraction of surviving organisms against time. The fraction of organisms surviving was plotted rather than the viable count itself, since the unavoidable variations in the initial count made comparison of graphs of viable counts more difficult. The actual initial count is stated on each graph.

The logarithmic plot assumed a slope which provided an index of the rate at which killing took place. This was expressed in terms of the 'decimal reduction time' (D.R.T.), i.e., the time required to effect a tenfold reduction in the viable count. The process did not however proceed to completion at a uniform rate: when a certain proportion of the cells had been killed the slope of the curve was diminished (e.g. Fig. 1, curve *C*) indicating that a small fraction of the cells were resistant to this treatment or at least exhibited a much longer D.R.T. than the remainder. This phenomenon, discussed below, necessitated expression of the results in terms of both the D.R.T. and the proportion of 'resistant' organisms. It was difficult to determine more than the order of magnitude of the latter, since exact evaluation was prevented by the preliminary period of shaking required before the 'initial' count. During this period destruction of the normal cells will proceed more rapidly than that of the 'resistant' organisms.

Course of the process

Fig. 1, *C*, illustrates a typical experiment with *Bacterium coli*. Up to 10 hr. killing proceeded in accordance with the exponential law, the D.R.T. being 2·1 hr., with the viable count decreased to *c.* 10^{-5} of its original value. Sterilization ceased at this stage, and little further fall in the viable count took place. These figures were confirmed in several replicate experiments performed on different occasions. The same D.R.T. and the same proportion of survivors were obtained with suspensions of only 1/10 the original density. No appreciable fall in the viable count was observed when the suspension was left in contact with the beads but not shaken (Fig. 1, curve *A*), and only a slight fall when shaking was carried out in the absence of the beads (Fig. 1, curve *B*).

The apparently high resistance of a small number of cells towards a process lethal to the majority was an unexpected finding. It was necesary to establish that this phenomenon was not due to some factor related to our technique (such as the protection of a small number of cells beneath the rubber lining of the stopper which closed the vessel in which shaking took place), though the constancy of the proportion of survivors for a given species made this explanation seem improbable. The phenomenon was investigated by shaking a suspension of *Staphylococcus aureus* for 12 hr.; this decreased the viable count from 10^9/ml. to a constant value of 1500/ml. The suspension was then transferred aseptically to another bottle containing fresh sterile beads and shaking continued for a further 9 hr. No significant fall in the viable count took place. At the same time a fresh (unshaken) suspension containing 2000 cells/ml. was shaken with beads in the usual way. In this control suspension the normal killing action was observed, except that the D.R.T. was considerably longer than that obtained with the much denser suspensions of *Staph. aureus* normally used (Table 1). The possibility that the 'resistant' cells owed their survival to protection by cell debris was also considered, though this seemed unlikely as the proportion of resistant cells was within wide limits independent of the initial density, and protection by debris would become apparent as soon as a substantial proportion of the cells had been disintegrated. The D.R.T., however, was found to remain constant until all but a minute fraction were destroyed. Moreover, when a suspension of *Staph. aureus* was shaken for 18 hr. (i.e. until the viable count became constant) and then reinoculated with a few drops of a dense suspension of fresh cells, the latter were killed at the normal rate when shaking was continued. It also seemed unlikely that cell multiplication could play any part. The generation time of a typical organism (e.g. *Bact. coli*) at the temperature of our experiments, even in an optimal medium, would be at least 2 hr., corresponding to a decimal increase time of 7 hr. When shaking was performed

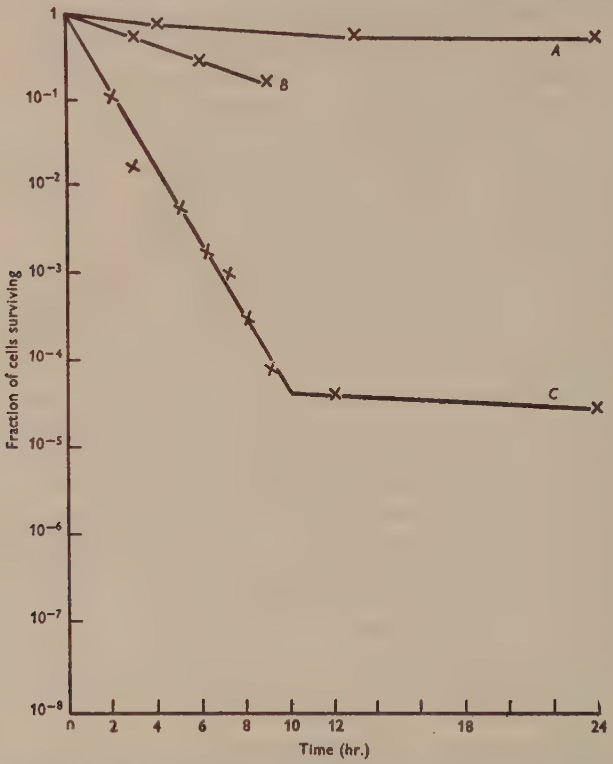

Fig. 1. Effect of shaking with and without beads. *Bact. coli*. *A*, control, standing with beads but not shaken; *B*, shaken without beads; *C*, shaken with beads. Initial count $10^{8.5}$ viable units/ml. in each case.

Table 1. *Relative resistance to mechanical destruction of normal and 'resistant' cells*

	Viable counts (organisms/ml.)	
Time (hr.)	*Staph. aureus* previously shaken for 12 hr.	Untreated control cells
0	1500	2000
3	1100	500
6	1300	190
9	1200	150

in broth, however, the 'resistant' cells did multiply more rapidly than they were destroyed; and once the normal, susceptible, cells were killed, the viable count increased slightly during the period 10–24 hr. (Fig. 2, curve *A*).

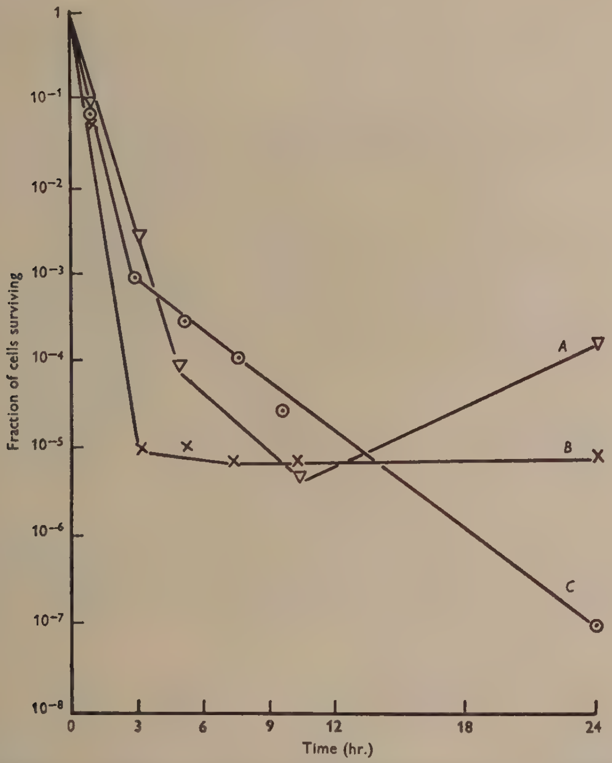

Fig. 2. Destruction of *Staph. aureus* in various suspending fluids. *A*, broth, initial count $10^{8.6}$/ml.; *B*, water, initial count $10^{7.4}$/ml.; *Ç*, saline, initial count 10^{7}/ml.

Resistance did not appear to be a genetic property. A suspension of *Staph. aureus* was shaken for 15 hr. and then subcultured on nutrient agar. As soon as growth was sufficient the organisms were harvested and again shaken for 15 hr. This was repeated twice without producing any change in either the D.R.T. or the proportion of resistant cells.

Factors affecting the killing of organisms

Size of beads. We found the two smaller sizes (diam. 0·26 and 0·13 mm. respectively) to be about equally effective (D.R.T. with *Staph. aureus* 1·4 hr.) and considerably more effective than the large size (diam. 0·86 mm.) which gave a D.R.T. of 2·8 hr. under the same conditions. The size of even the smallest beads is still large compared with that of the bacteria.

Optimal proportion of beads to suspension. This was found to be approximately equal weights of each.

Suspending fluids. Three were investigated, viz. water, saline and nutrient broth (Fig. 2). Water and saline gave approximately the same D.R.T. but the resistant organisms were slowly destroyed in saline whilst able to survive indefinitely in water. In broth considerable frothing took place. The D.R.T. was increased and the resistant cells, in this nutrient medium, were able to multiply faster than they were destroyed. Fig. 2 is based on experiments performed some time after the bulk of this work: a shaker operating at 450–500 strokes/min. was in use, and a decreased D.R.T. was obtained.

Table 2. *Decimal reduction time* (D.R.T.) *and proportion of resistant cells in various organisms shaken with minute glass beads*

Organism	D.R.T. (hr.)	Proportion of resistant cells
Staph. aureus	1·4	1 in 1,000,000
Enterococcus	2·2	1 in 1,000
Pneumococcus	2·45	Not estimated
Corynebacterium xerosis	1·5	Not estimated
B. subtilis	2·5	1 in 100,000
B. mesentericus	4·1	1 in 100
B. anthracis	2·7	1 in 2,000
Myco. smegmatis	3·2	Not estimated
Bact. coli	2·1	1 in 100,000
Shigella sonnei	2·15	1 in 100,000
Salm. paratyphi B	2·0	1 in 100,000
Brewers' yeast	7	Not estimated

Investigation of various organisms

The relative susceptibility of various organisms is illustrated by Table 2 and Fig. 4. With *Staph. aureus* (Oxford 'H' strain) four replicate experiments gave D.R.T. of 1·4, 1·4, 1·35, 1·45 hr. respectively (two of these experiments are illustrated by Fig. 3, curves *A* and *B*). Curve *C*, Fig. 3, represents an experiment with the shaker operated at 450–500 strokes/min. The proportion of resistant cells was found to be the same, but the D.R.T. was reduced to 0·45 hr. and the 'resistant' cells themselves were slowly destroyed. Gram staining of *Staph. aureus* after several hours' shaking showed a few Gram-positive cells, the debris being Gram-negative. Staining by the Indian ink capsule method revealed the presence of 'ghosts'. *Myco. smegmatis*, with a D.R.T. of 3·2 hr., was the most resistant bacterium studied; the 18 hr. period of shaking was not sufficient in this case to determine whether any cells were resistant. The disintegrated organisms were not acid-fast. After 18 hr. no intact bacilli could be seen in a stained film, though the viable count was still 10,000/ml.

Yeast proved comparatively resistant to this treatment (D.R.T. approx. 9 hr.). The lower initial count in this experiment (Fig. 4, curve *A*) was due to the standardization of the suspension to the same opacity as the bacterial suspensions used. It may be emphasized that the technique used was that found to be most effective for the destruction of *bacteria*; possibly a more rapid destruction of yeast might be obtained under different conditions.

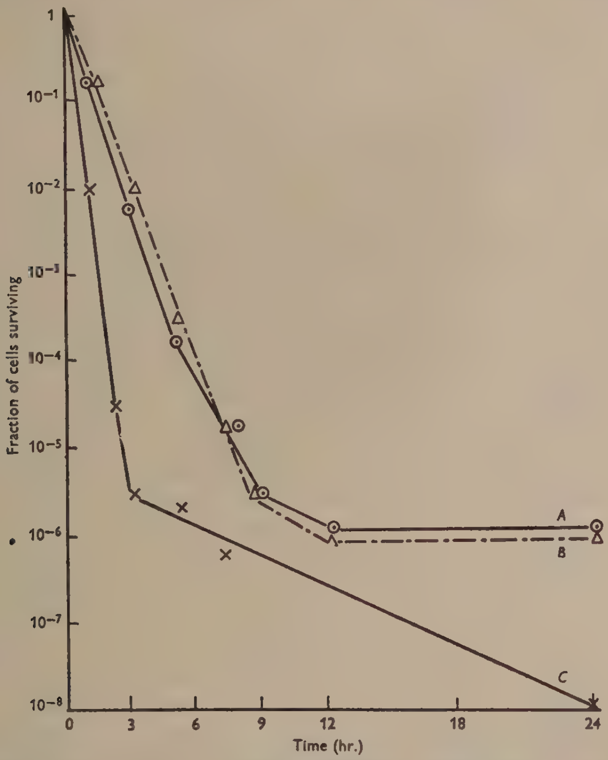

Fig. 3. *Staph. aureus. A* and *B* replicate experiments. Initial counts $10^{9 \cdot 3}$ and 10^{9}/ml. respectively; *C*, later experiment with more efficient shaking. Initial count $10^{8 \cdot 5}$/ml.

Biological factors determining resistance

The results described above indicate both that considerable differences may exist between different species of micro-organisms, and also that a few members of an apparently homogeneous culture may differ sharply from the rest in their resistance towards mechanical disintegration. In the experiments to be described we attempted to correlate these findings with differences in the other biological characteristics of the species and individuals concerned.

Resistance of spores to staining. This, like resistance to shaking, might be regarded as a property of the cell-wall. When *B. subtilis* is grown on a rich medium, such as blood-agar, the spores are readily stained by the Ziehl-Neelsen technique: on a relatively poor medium, e.g. 0·05 % peptone-agar, the spores are larger and less readily stained. Suspensions prepared from spores grown on these two media did not differ in D.R.T.

Resistance of vegetative cells to heat. A suspension of *Staph. aureus* was shaken for 15 hr., the viable count becoming constant at 1000–2000/ml. A control was prepared by sterilizing a similar suspension (60 min. at 60°) and adding fresh unshaken organisms to give a count of 1000–2000/ml. Both suspensions were then placed in a bath at 60° and a loopful from each stroke-inoculated on nutrient agar at 5 min. intervals. Both suspensions became sterile after 35–40 min. There was therefore no correlation between heat- and mechanical-resistance in this organism.

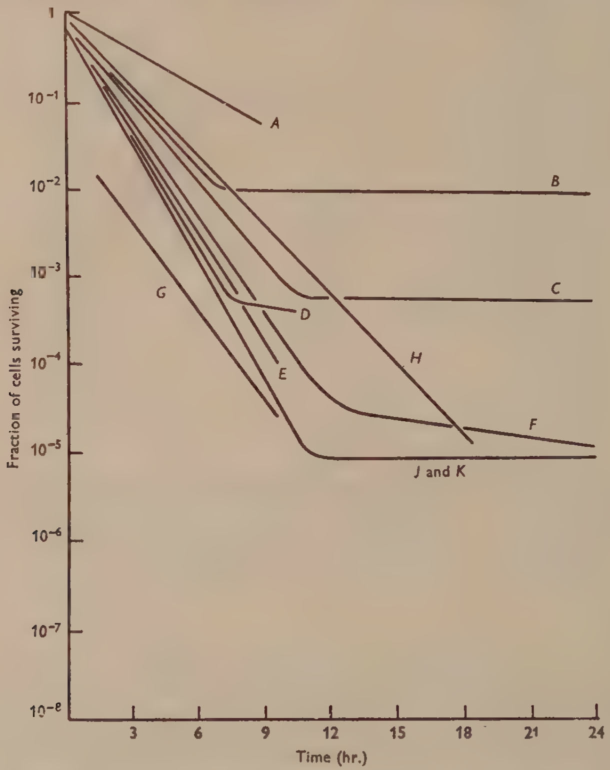

Fig. 4. Susceptibility of different species to destruction by shaking with minute glass beads. *A*, Brewers' yeast, initial count 10^5/ml.; *B*, *B. subtilis*, initial count 10^9/ml.; *C*, *B. mesentericus*, initial count 10^8/ml.; *D*, Enterococcus, initial count 10^9/ml.; *E*, Pneumococcus, initial count 10^9/ml.; *F*, *B. anthracis*, initial count $10^{8\cdot3}$/ml.; *G*, *Corynebacterium xerosis*, initial count $10^{7\cdot3}$/ml.; *H*, *Myco. smegmatis*, initial count $10^{9\cdot3}$/ml.; *J*, *Shigella sonnei*, initial count 10^8/ml.; *K*, *Salm. paratyphi B*, initial count $10^{8\cdot5}$/ml.

Penicillin resistance. Cells of *Staph. aureus* and *Shigella sonnei* which were resistant to shaking were normal in their sensitivity to penicillin.

Age of culture. A 4 hr. culture of *Staph. aureus* gave a D.R.T. of 2·3 hr., compared with 1·4 hr. for the usual 16–20 hr. cultures. Young cultures are generally regarded as being more susceptible to injurious influences than mature cultures, but young cells are also known to be abnormally large and we found large organisms to be more resistant to our treatment—cf. yeast and the large-capsule strains of *Aerobacter* spp. described below.

Capsules. Through the courtesy of Dr J. P. Duguid, of this Department, we examined three strains of *Aerobacter aerogenes*: Aerobacter I, a strain with a capsule of about 1μ radius; Aerobacter IA, the same organism grown on a medium (agar containing 1 % glycerol and 0·1 % peptone) on which it produced a capsule 5–10μ radius; Aerobacter II, with a small capsule, $0{\cdot}25\mu$ radius. The size of the capsules was measured in Indian ink preparations. There appeared to be a marked correlation between capsule size and resistance. The D.R.T. was 2·7 hr. for Aerobacter II, 4·8 hr. for Aerobacter I, while Aerobacter IA was completely resistant. The suspension of the latter was highly viscous (η 11·2 relative to water at 37°) and it was possible that the viscous material protected the organism. For purposes of comparison a suspension of *Staph. aureus* was shaken in a viscous medium (hog gastric mucin, η 18·3 relative to water at 37°). The D.R.T. was 2·6 hr., compared with 1·4 hr. for the control shaken in saline. The viscosity of the solution could thus play a part in protecting the organisms, but it cannot account for the resistance of the highly capsulated *Aerobacter* spp.

Table 3. *Proportion of smooth colonies among resistant and normal organisms*

	Shigella sonnei	*Salm. paratyphi B*
Normal	**16 smooth in 405 (4·0 %)**	**8 smooth in 338 (2·4 %)**
Resistant	**41 smooth in 272 (15 %)**	**31 smooth in 300 (10·3 %)**

The capsulated pneumococcus gave a D.R.T. 2·45 hr. compared with 1·4 hr. for the non-capsulated *Staph. aureus*. The enterococcus, also non-capsulated, gave a D.R.T. of 2·2 hr. however. This organism is known to be abnormally resistant to other destructive agents, e.g. heat. But even if the protective action of a capsule be established, we have no evidence whether this effect is specific or is due only to the larger size of the organism. Nor have we established whether the resistance of large organisms is real or due to our application to them of a technique developed with normal-sized non-capsulated bacteria (*Bact. coli*, *Staph. aureus*). The highly resistant survivors remaining after shaking normally susceptible organisms exhibit no abnormality in either size or capsulation.

Colony form. In *Shigella sonnei* and in *Salm. paratyphi B* the resistant organisms gave a higher proportion of smooth colonies than the initial suspensions (Table 3). But though the differences were statistically significant ($t > 4$ in both cases), it is difficult to regard the smooth organisms as playing any part in the phenomenon of resistance. A comparison of the relative resistance of suspensions of rough and smooth organisms failed to reveal any significant differences.

DISCUSSION

Our results confirm the conclusion of Curran & Evans (1942) that agitation with small inert particles may exert a powerful bactericidal action. But sterility is not readily obtained by this method: a small but definite proportion of the organisms shows resistance of an order different from that of the remainder of the culture. These 'resistant' cells did not differ from the normal cells in any of the other characteristics which we investigated. Curran & Evans (1942), whose investigations were confined almost entirely to spores, assessed the efficiency of mechanical destruction by the number of surviving organisms after 5 hr. shaking. This, however, is a function of several factors: the initial rate of killing, the proportion of resistant cells, and the rate at which the latter are killed. As the number of survivors after 5 hr. is the only figure cited in the majority of their experiments, comparison of their results with ours is not possible in most cases.

Our investigations with different species suggest that capsules may exert a protective influence, but it is not certain whether this is due solely to the increased size of the capsulated organism. Nor do we know whether the conditions of our experiments, developed with non-capsulated bacteria, are the most suitable when dealing with larger organisms. No correlation was found between resistance to mechanical destruction and the other biological characteristics of the organisms investigated. Whilst within one group of three closely related organisms—the Gram-negative bacilli—identical results were obtained, within another group of three biologically related organisms—the aerobic sporing bacilli—there was wide variation in resistance.

We are much indebted to Prof. T. J. Mackie for his interest in this work which was performed during the tenure by one of us (H. K. K.) of the Lewis Cameron Teaching Fellowship of Edinburgh University.

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Nuclear changes in *Bacillus anthracis* and their Relation to Variants

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SUMMARY: The nuclear changes leading to spore formation in *Bacillus anthracis* were investigated in 35 strains. Fusion of four chromatinic bodies into one mass and its subsequent break up into four bodies was observed. One of these became incorporated in the spore; the other three disintegrated. Some cells showed no fusion before spore formation; in these one chromatinic body was incorporated in the spore and the other three disintegrated. The percentage of cells in which fusion occurred varied with the strain. Rough colony strains showing a high incidence of fusion tended readily to throw smooth colony variants. Strains showing a low incidence of fusion were stable in this respect. The incidence of variants of other types bore no relation to the incidence of nuclear fusion in the strain.

Nuclear structures in the spore-forming aerobic bacilli were described by Nakanishi (1901), Badian (1933), Stille (1937), Piekarski (1938), and in greater detail by Robinow (1942), who studied them particularly in spore germination and in the early logarithmic phase of growth. Badian described the fusion of the nuclear elements into one mass and its subsequent breaking up into four parts before spore formation; he recognized that one body entered the spore while the remaining three disintegrated. Klieneberger-Nobel (1945) observed fusion of chromatinic structures both in aerobic and anaerobic spore-forming organisms, and also in a non-spore-forming organism, *Sphaerotilus natans*. In later papers (1947*a*, *b*) she described the changes occurring in myxobacteria and actinomyces, and showed that fusion of chromatinic structures occurred also among them. It was suggested both by Badian and Klieneberger-Nobel that this fusion might represent an autogamic mechanism.

MATERIALS AND METHODS

The following 35 strains of *Bacillus anthracis* were examined.

A. National Collection of Type Cultures: nos. 109 (Paddington VI); 1328; 1712 (Pasteur's Second Vaccine); 1607; 2620 (Hankow Hide); 4991, 5130, 5249, 7200.

B. Strains isolated from a recent shipment of sheep hides, many of which were contaminated, causing a human infection: strains A, C1, CC2, H2, H3, H5, H7, H8, F1, H3 F2, mouse F, mouse 5.

C. Strains isolated from the lesions of human and bovine infections: Hillsborough, Newtownards, Carrickfergus, R.V.H., Tinsdale, McLean 1A, McLean 2, Lindsay, Davis.

D. Strains maintained in this laboratory: A/stock, S/stock, Dean, 2160, 2161.

The media used were Lemco broth, pH 7·6, and Lemco agar (2% agar), pH 7·6.

The staining methods described by Robinow (1944) were used to demonstrate the nuclear structures. For the study of spore germination and of young cultures up to 4 hr. old, the agar was cut into strips and mounted on cover-slips, with the organisms outwards, and fixed in the vapour of 2 % osmic tetroxide for 3–5 min. A second cover-slip was then placed on the agar surface, and the two cover-slips with the agar between them immersed in 30 % ethanol for 2–3 hr. The second cover-slip, bearing most of the organisms upon it was then removed and placed for a few minutes in Schaudinn's fixative, then in N-HCl at 56° for 7 min., washed, and stained with Giemsa's stain (0·15–2 ml. phosphate buffer, pH 6·2) for 10–15 min. For the details of this technique, I am indebted to Dr C. F. Robinow (personal communication).

For older cultures, a portion of growth was emulsified in a small drop of water, allowed to dry, and immediately fixed in osmic tetroxide vapour, hydrolyzed and stained as above. All preparations were made on cover-slips. It was found that a more transparent stain was produced by diluting the Giemsa stain with a mixture of 2 vol. horse serum and 1 vol. M/200 phosphate buffer, pH 6·2.

Fat inclusions were stained by the method of Burdon, Stokes & Kimbrough (1942).

All preparations were examined and photographed mounted in water or in the staining solution.

Optical equipment. The optical equipment consisted of a 2 mm. Leitz apochromatic objective, N.A. 1·32, with compensating eyepieces × 12 for photography and × 20 for visual work, and a Watson 'Holoscopic' oil immersion condenser, N.A. 1·3. Critical illumination was used throughout. A green filter was used both for photography and for visual work. It was impossible to resolve some of the structures without oil immersion of the condenser.

RESULTS

The changes found in spore germination confirm the results of Robinow (1942). The spores of *B. anthracis*, however, take 60–75 min. to germinate, a much longer time than that taken by the spores of his strain of *B. mycoides*.

In the earlier stage of logarithmic growth the rods contain dumb-bell-shaped bodies which divide at right angles to the long axis of the cell (Pl. 1, figs. 1, 2; Fig. 1A). When they separate they leave between them a single thread of chromatin which later ruptures. In the rough colony variants the chromatinic bodies are widely separated. In the smooth colony variants they are closer together, and the intercellular septa occur at an earlier stage than in the rough phase.

In the later logarithmic growth stage, after about 5–5½ hr. growth, 'vacuoles' appear in between the dividing chromatinic bodies; and now, instead of a single chromatin thread joining the separating bodies, two threads can be seen (Pl. 1, fig. 3; Fig. 1B), which are separated by the 'vacuole'. The nature of this 'vacuole' is not certain. Vacuoles may often be seen by dark-ground illumination after about 5 hr. growth in a position corresponding to that of the spaces seen in the Giemsa-stained preparation. They often contain a small granule in violent Brownian movement, showing that the contents are fluid. In many strains fat globules can be stained by Sudan Black in 5 hr. cultures corresponding in position to the vacuoles seen in dark ground and in the Giemsa-stained preparations.

From 5½ to 10 hr. of growth (the time varying from one strain to another, but fairly constant for the same strain on the same medium) four (occasionally eight) chromatinic bodies become joined end to end and then fuse into a single chromatin rod in the long axis of the cell. At this stage the vacuoles disappear (Pl. 1, fig. 4; Fig. 1C–E). Fusion occurs only in a certain percentage of the cells; with some strains, in less than 1 %, with others, in over 95 %. Just before this stage, each cell contains four chromatinic bodies, surrounded by a cell membrane; a few cells contain eight. The cells which do not show this change remain as before, with their chromatin structures in pairs separated by vacuoles. As a rule, the axial chromatin rods remain unchanged for at least 45 min.: in one strain (N.C.T.C. 1328) they persisted for nearly 3 hr. Vacuoles then appear in the rods (Pl. 1, figs. 5–7; Fig. 1F, G) which break up to form four chromatinic bodies in two pairs, the members of each pair separated by a vacuole. Around any one of the four chromatinic bodies an eosinophil substance is laid down, usually around the terminal body (Pl. 2, fig. 8; Fig. 1G; cf. the diagrams of Badian and Klieneberger-Nobel). A limiting membrane around this eosinophilic area could not be seen. This eosinophil substance increases in size and becomes the spore body. Inside it the chromatinic body becomes orientated into the long axis of the cell, and then becomes spherical in shape. It increases in size, until the whole spore body becomes so densely basophilic that no internal structure can be distinguished (Pl. 2, figs. 9–13; Fig. 1H–J). The appearance is similar to that seen in one stage of the germinating spore. The basophilic material then clears and the chromatinic body appears lying on the surface of the spore body. Meanwhile, the three remaining chromatinic bodies disintegrate, and eventually the rest of the cell disappears leaving the free spore (Pl. 2, fig. 14; Fig. 1K, L). The cells in which fusion of nuclear substance does not take place go on to spore formation in the same way.

Attempts were made to induce fusion of nuclei in strains in which this rarely happened. Following a suggestion of Dr Klieneberger-Nobel, cultures at various stages of growth of the strain 'Carrickfergus' were examined after 3 hr. at 0°. Clumping of the chromatinic bodies occurred in the early stage of logarithmic growth (Pl. 2, fig. 15; Fig. 1M), but at the stage immediately before spore formation, when fusion might have been expected, no fusion occurred, possibly because of the vacuoles between them.

When this strain was grown on Lemco agar containing 1/2000 (w/v) $CaCl_2$, as recommended by Bordet & Renaux (1930) for production of asporogenous variants, about 15 % of fusion cells occurred, instead of the usual 1 %. Similarly, the strain Hankow Hide, normally producing about 40 % fusion cells, produced over 90 % when grown on this medium.

For the estimation of the percentage of cells in which nuclear fusion took place, the Lemco agar plate was heavily and evenly inoculated over an area of at least 2 sq.in., so that the cells in preparations made from the centre of this area were as nearly as possible at the same stage of development. In strains showing almost 100 % nuclear fusion, few preparations were necessary; in strains whose cells showed little fusion, very many preparations were made to ensure that fusion was not taking place. The figures in Table 1 are those of the preparations showing the highest incidence of fusion cells found in the strain in question.

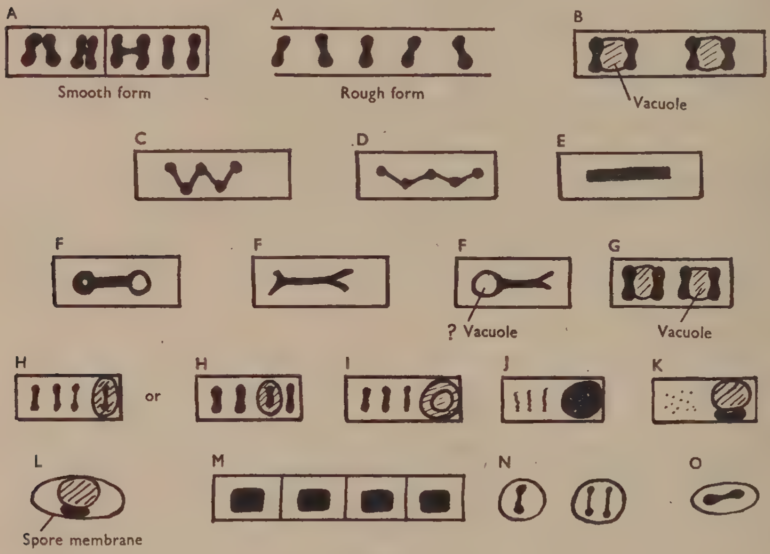

Fig. 1.

All the strains were inoculated into 100 ml. of Lemco broth, pH 7·6, and incubated at 37°. The cultures were thoroughly shaken after 10 and 17 days' growth and plated out to give single colonies. Between 50 and 100 colonies of each strain were examined. The 17-day period was chosen because variation takes place in most strains of *B. anthracis* by this time; and if cultures in a large volume of broth are incubated longer, motile variants sometimes appear. When this happens all or nearly all of the colonies found on plating are smooth colony motile variants; the other forms are overgrown in the flasks. In estimating the incidence of cells showing fusion the difficulty was greatest in strains showing an incidence between 10 and 90 %; some cells are seen in which it is hard to decide whether or not an early or late stage of fusion is taking place. Over this range the percentage error is probably at least ±10 %. In the table the letter 'R' indicates the rough medusa-head colony variant and 'S' the smooth, usually avirulent, colony variant. 'RS' indicates the intermediate form.

Bacteriophage was present in the strains C1, H2, and F1. The strain C1,

Table 1. *The incidence of nuclear fusion in variants of* Bacillus anthracis

Strain	Incidence of fusion (%)	Type of colonies after 10 days' incubation	Type of colonies after 17 days' incubation
N.C.T.C. 109	70	All R	RS 20 % R 80 %
N.C.T.C. 1328	30	All R	All R
N.C.T.C. 1607	4	All R	All R
N.C.T.C. 1712	95	All R	S 52 % R 48 %
N.C.T.C. 2620	40	All R	A few coccal variants Remainder R
N.C.T.C. 4991	90	All R	S 12 % RS 67 % R 15 %
N.C.T.C. 5180	1	All R	S 1 % RS 1 % Remainder R
N.C.T.C. 5249	95	All R	S 40 % RS 33 % R 27 %
N.C.T.C. 7200	20	All R	All R
A	1	All R	All R
CC2	10	RS 1 % R 99%	RS 5 % R 95 %
H3	50	S 50% R 50 %	RS 60 % R 40 %
H5	95	RS 10 % R 90 %	S 30 % R 70 %
H7	80	All R	S 20 % RS 35 % R 45 %
H3 F3	60	S 30 % R 70 %	S 30 % R 30 %
Mouse F	95	RS 1 % R 99 %	RS 30 % R 70 %
Mouse 5	5	All R	All R
Hillsborough	1	All R	All R
Newtownards	10	Ghost colonies (R) 50 % R 50 %	Ghost colonies 70 % R 70 %
Carrickfergus	2	All R	All R
R.V.H.	20	All R	Coccal variants 75 % R 25 %
Tinsdale	5	All R	All R
McClean IA	10	Small smooth 1 % R 99 %	1 coccal variant Remainder R
McClean II	40	All R	S 65 % R 35 %
A/stock	50	All R	Coccal variants only
S/stock	25	All R	All R
Lindsay	75	Coccal variants 65 % R 35 %	S 15 % R 85 %
Dean	70	All RS as original	All S
2160	25	All R	S 10 % R 90 %
2161	60	1 smooth colony Remainder R	S 4 % R 96 %
Davis	95	RS 5 % R 95 %	All R

with an incidence of fusion of 95 % regularly produced about 50 % of smooth colony variants after 24 hr. incubation. The strains H 2 (fusion incidence 1 %) and F 1 (fusion incidence 5 %) did not throw any smooth colony variants from broth cultures. (Smooth colony variants were obtained on plate culture from the strain F 1 in the presence of bacteriophage of high titre which was specific for the rough colonies (N. C. Graham, personal communication).)

These results show that the occurrence of smooth colony variants in rough colony cultures is associated with a high incidence of cells showing fusion of the chromatinic bodies and that stability is associated with a low incidence. The only exceptions were the N.C.T.C. strain 5130, where one smooth and one RS colony were produced, and the strain 'Davis', where hardly any variation occurred. The occurrence of coccal and ghost-medusa colonies is not related to the incidence of nuclear fusion.

DISCUSSION

There can now be little doubt that the structures seen in bacteria stained by the osmic acid HCl-Giemsa method represent the nuclear apparatus. They are present in all organisms examined which are large enough to show internal structure under the light microscope (Robinow, 1942, 1944, 1945 and Klieneberger-Nobel, 1945, 1947 *a*, *b*). The distribution and appearance of these structures is similar by different staining methods: Nakanishi (1901), using a 'vital' method with methylene-blue; Dobell (1911) and Badian (1933), fixing with osmic acid or formalin, staining with Giemsa, and differentiating with 1 % eosin solution; Stille (1937), Piekarski (1940), and Neumann (1941) using the Feulgen technique; Robinow and Klieneberger-Nobel using the osmic acid HCl-Giemsa method; all saw essentially the same structures.

The Feulgen stain in all other plant and animal tissues is specific for the desoxyribonucleic acid which is found only in nuclei and which is associated with the presence of genes. Vendrely & Lehoult (1946) have estimated the dry-weight percentage of ribonucleic and desoxyribonucleic acids and protein in staphylococci, *B. anthracis* and *Escherichia coli*. They found that the amount of desoxyribonucleic acid is in accord with the size of the nuclei described by Robinow, but that the nuclei of Stille and of Piekarski appear too small in their photographs. This may be due to the use of dehydrated and cleared preparations by Stille and Piekarski, whereas Robinow's preparations were photographed mounted in water.

The spore contains one chromatinic body; the coccal variant of *B. anthracis* contains a single nuclear body (Pl. 2, fig. 16; Fig. 1N) and in the phase of growth of *Proteus vulgaris* just before swarming occurs, forms containing one chromatinic body occur (Klieneberger-Nobel, personal communication). The cells of *Esch. coli* and *Proteus vulgaris* (Robinow, 1944) and the cells of *Corynebacterium diphtheriae* in old cultures contain only one nuclear body (Pl. 2, fig. 17; Fig. 1O). It seems, therefore, that the bacterial individual must be considered as having a single nuclear body which may reasonably be considered as a chromosome; and that the rod form of young cultures of species like *Esch.*

coli and *B. anthracis* consists of several individuals still sharing a common cytoplasm and cell membrane. So the fusion cell of *B. anthracis* represents the fusion of four individual organisms which have arisen by two divisions from the same ancestral individual.

Appearances suggesting nuclear fusion are seen in the illustrations of Nakanishi (1901) and Dobell (1911), in the aerobic and anaerobic spore-formers. They have been more fully described by Badian (1933) and Klieneberger-Nobel (1945, 1947*a*, *b*) in aerobic and anaerobic spore-forming organisms, myxococci and the aerobic actinomycetes. In a motile Gram-negative non-sporing bacillus Rosca (1937) described cells with *X* and *Y* chromosomes or nuclei which underwent fusion to form a diploid cell. His evidence is not convincing. Stapp (1942) described in *Pseudomonas tumefaciens* the formation of aggregates of rod forms like the spokes of a wheel with fusion of cytoplasm and chromatin in the centre, and their subsequent separation. His photographs are quite convincing. Zygospore formation has been described by Mellon (1925) in a rough strain of *Esch. coli*, but he did not demonstrate fusion of chromatin. The work of Lederberg & Tatum (1946) and of Tatum & Lederberg (1947) suggests that fusion of cells of one strain of *Esch. coli* may occur with those of another. They think that this does not necessarily occur in all strains, and that it may occur only rarely. It seems, therefore, that some form of fusion mechanism occurs widely among bacteria.

Lindegren (1935) postulated on theoretical grounds that a bacterial cell, having life, must possess genes arranged in a string that splits longitudinally in cell division. This linear arrangement of genes is probably represented by the dumb-bell-shaped chromosome. When the four chromosomes fuse, there may be an opportunity for interchange of position of genes on the chromosome. Change in the relative position of genes on the chromosome has been suggested by Darlington (1937) as a mechanism of variation in *Drosophila, Chorthippus* and *Tulipa*. There can, of course, be no doubt that change in the gene substance itself is also a mechanism for production of mutants.

The appearance when the fusion cylinder breaks up (Pl. 1, figs. 5–7) is reminiscent of the appearance of the chiasmata in the diakinesis stage in meiosis of higher plants and animals; but it is possible that this appearance is due to the presence of the developing vacuole, and that it has no other significance.

The results suggest the importance of nuclear fusion as an autogamic process for the production of rough colony → smooth colony → motile variants. It is possible that the other variant colony types (coccal, ghost medusa, dwarf medusa, extreme rough) arise by means of gene mutation only; but this has yet to be proven.

I am greatly indebted to Dr C. F. Robinow, Dr N. C. Graham, and Dr E. O. Bartley for their interest, help, and criticism in this investigation, and to Dr Robinow also for details of technique and photography.

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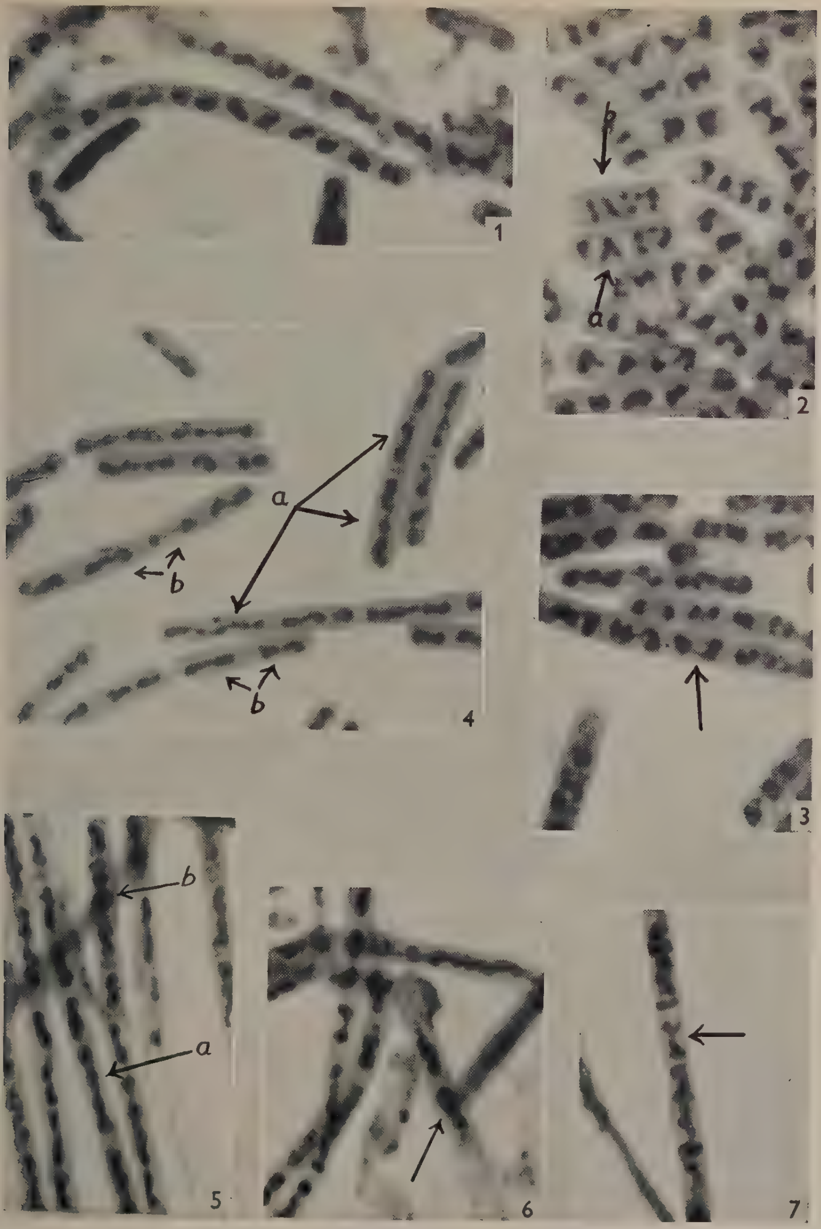
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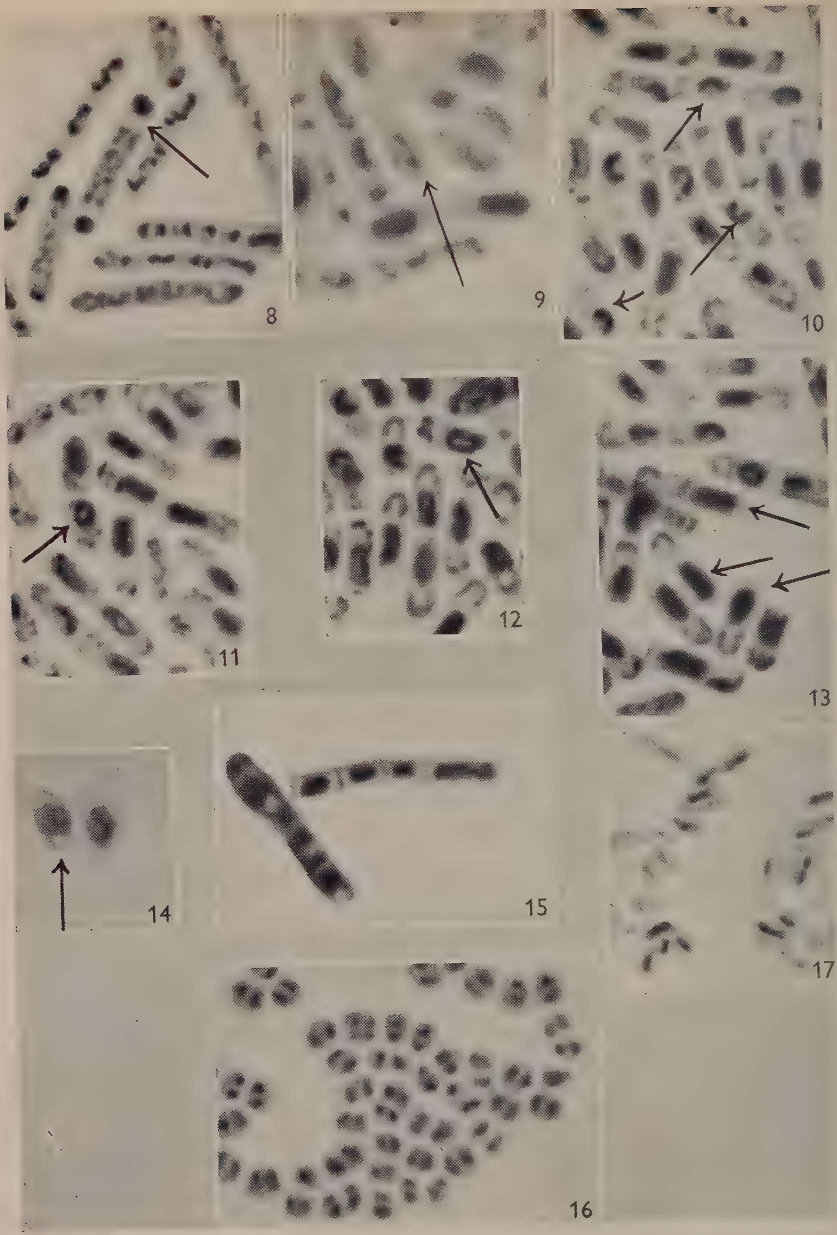
Figs. 1–7

T. H. Flewett—Nuclear changes in *Bacillus anthracis* and their relation to variants. Plate 1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Figs. 8–17

T. H. FLEWETT—NUCLEAR CHANGES IN *BACILLUS ANTHRACIS* AND THEIR RELATION TO VARIANTS. PLATE 2

EXPLANATION OF PLATES

PLATE 1

All photographs were of cover-slip preparations mounted in water. Magnification ×4000.

Fig. 1. Strain Hankow Hide, rough colony type, after 3½ hr. incubation, showing widely spaced chromosomes.

Fig. 2. Strain C1, smooth colony type, after 3½ hr. incubation, showing (*a*) incomplete division of chromosomes, and (*b*) divided chromosomes lying close together. The chromosomes are more closely spaced than in Fig. 1. Photograph by Dr C. F. Robinow.

Fig. 3. Strain Carrickfergus, after 4½ hr. incubation, showing separating chromosomes connected by two threads of chromatin enclosing a vacuole.

Fig. 4. Strain Hankow Hide, after 7 hr. incubation on agar containing $CaCl_2$ 1 : 2000 (w/v), showing (*a*) chromosomes joined end to end, and (*b*) complete fusion.

Fig. 5. Strain and medium as in Fig. 4; 7½ hr. incubation. Stages in the breaking up of the fusion cylinders: (*a*) a long form, probably derived from eight chromosomes, and (*b*) a short form derived from four chromosomes.

Figs. 6 and 7. Strain and medium as in Fig. 4, showing appearances suggestive of chiasma formation.

PLATE 2

Fig. 8. Strain Carrickfergus, 8 hr. incubation on ordinary agar, showing an early stage of the spore; the remaining chromatin material is indistinct.

Fig. 9. Strain C1. A later stage than Fig. 8. The spore is larger than in Fig. 8 and the chromosome is in the long axis.

Fig. 10. Strain Hankow Hide, 8½ hr. incubation. The chromosome is bent into a horse-shoe shape.

Figs. 11 and 12. Later stages of Fig. 10 showing expanding rings of chromatin.

Fig. 13. Completely basophilic spores, similar to those seen in one stage of germination.

Fig. 14. Strain C1. Resting spores, hydrolyzed at room temperature, by oblique illumination, showing the spore chromosome and the outer spore membrane.

Fig. 15. Strain Carrickfergus after 3½ hr. incubation at 37° and 1 hr. at 4° showing clumping of nuclear material.

Fig. 16. Strain Tinsdale. Coccal variants, showing the single chromosome. Dividing forms show two chromosomes. 3 hr. incubation. Photograph by Dr C. F. Robinow.

Fig. 17. *C. diphtheriae*, 10-day culture on Neill's blood-tellurite medium, showing single chromosome, photographed in light of the $435 \cdot 8\mu$ band of the mercury arc.

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The Egg-Yolk Reaction of Aerobic Sporing Bacilli

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SUMMARY: Among a large number of strains of aerobic sporing bacilli only *Bacillus cereus*, *B. mycoides* and, to a lesser extent, *B. anthracis* were able to produce turbidity and formation of a curd in saline extract of egg-yolk. None of the other species tested, namely, *B. alvei*, *B. alcalophilus*, *B. brevis*, *B. carotarum*, *B. circulans*, *B. coagulans*, *B. fusiformis*, *B. licheniformis*, *B. macerans*, *B. megatherium*, *B. pumilus*, *B. polymyxa*, *B. orpheus*, *B. repens* and *B. subtilis* caused any opalescence in the yolk medium. The yolk reaction was due to the action of phospholipinase produced by the organisms. As in the case of *Cl. welchii* α-toxin, the yolk curd-forming activity of *B. cereus* and *B. mycoides* also was associated with haemolytic activity. The substances responsible for these activities appear to be similar, and may be identical. The yolk reaction has proved useful for the rapid identification of *B. cereus*, as it is more specific than any of the other distinguishing tests hitherto employed. The three positive organisms, *B. cereus*, *B. mycoides* and *B. anthracis*, have been considered by previous workers on the grounds of morphology and antigenic structure to be closely related.

Nagler (1939) described the effect of *Cl. welchii* cultures and toxic filtrates on human serum in producing an opalescence and curding (Nagler reaction); later Macfarlane, Oakley & Anderson (1941) found that egg-yolk saline (a lecitho-vitellin emulsion) is even more sensitive than human serum (the lecitho-vitellin (L.V.) reaction). Macfarlane & Knight (1941) then showed that the production of opalescence in egg-yolk emulsion was due to the action of a lecithinase, which split phosphocholine from lecithin, in the *Cl. welchii* toxic filtrates which was probably identical with the α-toxin. Since then, the Nagler reaction and the lecitho-vitellin (L.V.) reaction have been extensively used for the titration of α-toxin of *Cl. welchii* (Nagler, 1939; van Heyningen, 1941*a*) and for the rapid identification of certain clostridia (Hayward, 1941, 1943).

It is interesting that Nagler (1939) observed a non-pathogenic aerobic spore-bearing bacillus which caused an opalescence in human serum like that caused by *Cl. welchii*; but the effect was not inhibited by *Cl. welchii* antitoxin. Crook (1942) and Hayward (1943) also mentioned that some aerobic bacteria caused similar opalescence. In the light of these observations we have sought for similar enzymes in the aerobic spore-bearing bacilli. The work started with a survey of the *Bacillus* group to ascertain which of them produced an egg-yolk reaction. A test of 260 identified strains, comprising 24 species, showed that only *B. cereus*, *B. mycoides* and some strains of *B. anthracis* were positive. In comparison with other characteristics generally used for the differentiation of the *Bacillus* group, the yolk reaction seems to be much more selective. One can hardly find any other characteristics which are confined to only two or three species of this very large group of organisms, and the test deserves the attention of taxonomists of the *Bacillus* group. We have investigated the specificity of the yolk reaction among aerobic spore bearers, the use of the yolk broth and yolk agar for the rapid identification of *B. cereus* and *B. mycoides*, and the possible correlation between the yolk reaction, haemolysis and phospholipinase activity. The properties of the phospholipinase have been studied in some detail and will be more fully reported in a subsequent paper.

METHODS AND MATERIALS

Cultures. For this investigation 260 previously identified strains and about 100 freshly isolated strains of the aerobic spore bearers were used. They included 24 species and many classical strains. Most of the cultures were identified according to Gibson & Topping (1938).

Media

Egg-yolk saline. Earlier workers used the yolk of one egg in 250 or 500 ml. saline. As the sizes of yolks were found to vary, a 5 % (w/v) yolk saline was used instead. It corresponds to one egg-yolk in 300–400 ml. saline, as egg-yolks usually vary from 15 to 20 g. The yolks from fresh eggs were weighed and diluted with physiological saline to the required volume. To every 100 ml. of the suspension about 2 g. of kieselguhr were added. After stirring, the mixture was filtered through paper pulp on a Buchner funnel, and then sterilized by passing through a Seitz EK filter. The solution was distributed in small bottles and kept in the refrigerator.

Yolk broth. Yolk broth (5 % w/v) was prepared the same way as yolk saline, except that nutrient broth was used instead of saline. It was distributed in tubes and flasks. The medium is perfectly clear by transmitted light and remains stable for weeks if kept sterile in the refrigerator. The yolk-broth tube is most useful for tests of pure cultures. Organisms that produce potent phospholipinase render the medium thickly milky after 8–10 hr. incubation. A thick curd usually appears on the top of the medium in 16–24 hr. Other organisms grow in it without any change of the medium. The difference can be seen in Pl. 1, fig. 1.

Yolk-agar plate. The yolk agar was prepared by mixing equal parts of the above 5 % yolk broth and 4 % nutrient agar at 45° with sterile precautions and immediately pouring into sterile Petri dishes. The resultant medium is simply an ordinary nutrient agar containing 2·5 % egg-yolk. The yolk-agar plate is mainly used for the isolation of phospholipinase-producing organisms, the colonies of which are clearly marked by a thick opaque zone usually extending several mm. from the colony. Pl. 1, fig. 2 shows how *B. cereus* can be readily isolated from soil by plating on egg-yolk agar. The yolk-agar plate can also be used for testing pure cultures. One plate can be conveniently used for seven cultures. Pl. 1, fig. 3 shows the different appearance of the colonies of positive and negative organisms. The width of the opaque zones serves as a rough indication of their relative activities. Negative organisms, instead of producing opalescence, usually render the medium clearer.

EXPERIMENTAL AND RESULTS

The specificity of the egg-yolk reaction in aerobic sporing bacilli

Two hundred and sixty identified strains of aerobic sporing bacilli were tested in yolk-broth and yolk-agar plates. The results (Table 1) show that the reaction was produced by *B. cereus*, *B. mycoides* and, to a lesser degree, by *B. anthracis*. Especially in *B. cereus*, the ability to react with the egg-yolk was a fairly constant characteristic. Of the 81 strains tested, 73 gave rise to a definite opaque zone extending beyond the colony within 24 hr.; of the other 8 strains, 7 produced opalescence in the medium immediately underneath the colony after incubation from 1 to 4 days. The N.C.T.C. strain 1599 W, labelled *B. cereus*, failed to show any opalescence in yolk agar, and was finally identified as *B. subtilis*. It was interesting to note that by plating on yolk agar, a strain of *B. cereus* was found to be a mixed culture of a positive *B. cereus* and a negative *B. licheniformis*. Another interesting finding is that the only strain of *B. megatherium* from the National Collection of Type Cultures found positive was identified by other tests as *B. cereus*. The reidentification of these strains has been confirmed by Mrs A. C. Stirling of the East Scotland and Edinburgh College of Agriculture.

Table 1. *Specificity of the egg-yolk reaction in aerobic sporing bacilli*

		Yolk-agar test			Yolk-broth test		
		Positive			Positive		
	No. of strains tested	In 24 hr.	In 1–5 days	Negative after 5 days	In 24 hr.	In 1–5 days	Negative after 5 days
B. cereus	80	73	7	—	72	5	3
B. mycoides	13	4	7	2	11	—	2
B. anthracis	20	5	15	—	5	12	3

Completely negative in both tests

	No. of strains tested		No. of strains tested		No. of strains tested
B. megatherium	23	*B. brevis*	5	*B. fusiformis*	8
B. circulans	8	*B. carotarum*	3	*B. alcalophilus*	2
B. subtilis	35	*B. alvei*	3	*B. coagulans*	4
B. pumilus	20	*B. macerans*	4	*B. repens*	2
B. licheniformis	18	*B. polymyxa*	3	*B. aminovorans*	1
B. freudenreichii	1	*B. firmus*	1	*B. orpheus*	1

Of the 13 strains of *B. mycoides* examined, 11 strains gave the reaction in yolk broth. The yolk-agar plate is not very suitable for *B. mycoides* because the opaque zone is usually obscured by the rapidly spreading growth of the organism, though reaction is evident from the increase of opacity in the medium underneath the centre of the colony. For the readily spreading *B. mycoides* yolk broth is more satisfactory.

With *B. anthracis* the reaction was much weaker. With most strains, the opalescence appeared only after 48 hr. incubation and seldom extended beyond the colonies. Some correlation between the colony appearance of *B. anthracis* and the yolk reactivity was noted. The strongly reacting strains were usually those with smoother colonies, which were rather like the colony of *B. cereus* than the typical medusa-head *B. anthracis* colony. Thus the avirulent variant H and the mucoid variant HM, which Dr P. Bruce White obtained from the typical strain 'Vollum', are much more potent in yolk reaction than their parent strain. Also, the smooth and smooth-mucoid variants that we obtained from a virulent strain Ellis by prolonged cultivation in broth, were found to be more active than the original strain. Furthermore, the two 'Pasteur vaccine' variants obtained from the National Collection of Type Cultures were also more active.

Table 2. *Variation of yolk reactivity among strains*

Species	Strain	Width of opaque zone beyond the colony (mm.) in 24 hr. yolk-agar culture	Time required to produce flocculation in yolk broth (hr.)	Arbitrary units of yolk-reacting substance in culture supernatant
B. cereus	1031	6	6	2·0
	945	4	6	1·0
	5806	4	8	0·35
	327	2	30	Undetectable
	909	*	72	Undetectable
B. mycoides	684	*	8	0·42
	681	*	8	0·28
B. anthracis	'Thorne'	3	12	Undetectable
	'HM'	3	12	Undetectable
	'Vollum'	*	60	Undetectable
	'Ellis'	*	60	Undetectable

* = The opaque zone confined to the area underneath the colony.

The substance reacting with egg-yolk could be demonstrated in the culture filtrate of most strains of *B. cereus* and *B. mycoides*, but not in any of the strains of *B. anthracis*. Like other biochemical activities, the yolk-reacting activity of these organisms also varies among strains (Table 2).

The mechanism of the yolk reaction

As with the α-toxin of *Cl. welchii* (Macfarlane & Knight, 1941), the yolk reaction of *B. cereus* and *B. mycoides* also was found to be due to the action of a phospholipinase. The yolk reactivity, measured by a modified van Heyningen's turbidimetric method (1941 *a*) ran in parallel with the phospholipinase activity, measured by the Macfarlane & Knight's method (1941). Differing from the lecithinase of *Cl. welchii*, the phospholipinase of *B. cereus* attacks both lecithin and kephalin, with the production of acid-soluble organic P but no inorganic P. The hydrolytic products of lecithin have been isolated and identified as neutral fat and phosphocholine. The properties of the enzyme, or enzymes, have been studied in some detail and will be reported more fully in a subsequent paper.

The association of yolk reaction with haemolytic activity

In *Cl. welchii*, the lecithinase activity, the yolk-reaction activity and the haemolytic activity were found to be closely associated. This association was also noted in *B. cereus* and *B. mycoides*. Strains producing the yolk reaction were always haemolytic. The opaque zone and the haemolytic zone produced in yolk agar and blood agar by 7 strains of *B. cereus* are compared in Pl. 1, fig. 4. The yolk-reaction activity and the haemolytic activity of the culture filtrates of a number of strains of *B. cereus* and *B. mycoides* also have been determined by the colorimetric methods of van Heyningen (1941*a*, *b*) and Herbert (1941). For most strains these two activities ran in close parallel. But a few strains had a much higher ratio of haemolytic activity. Thus the culture filtrate of strain 720, which showed only trace of yolk reactivity, was fairly haemolytic. The possibility of a second haemolysin, unrelated to the yolk-reacting substance, is under investigation. Although there is no direct evidence to show that haemolysis is due to the phospholipinase activity, indirect evidence strongly suggests that part, if not all, of the haemolysin of *B. cereus* and *B. mycoides* is closely correlated with the yolk-reacting substance or phospholipinase. An interesting observation is that both haemolysis and the hydrolysis of lecithin by culture filtrates of *B. cereus* are inhibited by normal serum (human, horse, ox, sheep, rabbit) in very high dilution.

B. anthracis generally is regarded as non-haemolytic, and lack of haemolysis has long been used as an important criterion for differentiating it from the saprophytic spore-bearing bacilli. However, it was observed that with most strains of *B. anthracis* examined, zones of haemolysis limited to the area underneath each colony developed after incubation for 2–3 days on sheep-blood agar. Thus haemolytic activity seems to be associated with the yolk-reaction activity, inasmuch as the opalescence produced by *B. anthracis* on yolk-agar plate also developed very slowly and seldom extended beyond the colony. It must be pointed out that although haemolysis and yolk reaction can be demonstrated in blood-agar and yolk-agar plates, neither haemolysin nor the yolk-reacting substance has ever been detected in the culture filtrate of even the most active strain of *B. anthracis* yet examined. The sheep erythrocytes used in the blood agar were washed because it was found that haemolysis produced by these organisms was strongly inhibited by normal serum. There is considerable variation in the sensitivity of erythrocytes from different species of animals.

DISCUSSION

The group of aerobic sporing bacilli comprises a very large number of species, the differentiation of which is still a problem for taxonomists. Although several classifications have been put forward by various workers (Bergey, Breed, Murray & Hitchens, 1939; Gibson & Topping, 1938; Smith & Clark, 1937;

Lamanna, 1940*a*, *b* & *c*), there is still much confusion and new tests would seem to be necessary for a satisfactory classification. With the simple egg-yolk reaction we have been able to pick out from this large group of organisms three species which have been suspected by previous workers to be closely related. *B. mycoides* is usually distinguished from other aerobic sporing bacilli solely by its characteristic filamentous colony. Apart from this feature, which is very liable to variation (Lewis, 1932; Gordon, 1940; Lamanna, 1940*b*), *B. mycoides* is almost indistinguishable from *B. cereus*. Their close relationship is further suggested by the finding of Lamanna (1940*b*) that many strains of *B. mycoides* possess the same spore antigen as that of *B. cereus*. Our finding that so far these are the only species in the large group of aerobic sporing bacilli found to produce this phospholipinase enzyme, strongly supports the view that they are related. The fact that *B. anthracis* can cause a rather weak and slow yolk reaction is also of interest. Although typical *B. anthracis* is quite distinct from *B. cereus* and *B. mycoides*, these latter are the organisms most likely to be confused with the anthrax bacillus; thus the strains of so-called '*B. anthracoides*', extensively studied by Grierson (1928), were mostly *B. cereus*. The results of our numerous biochemical tests support the opinions based on morphological and physiological studies of other workers, viz. that *B. cereus* and *B. mycoides* are closely related to *B. anthracis*.

The egg-yolk reaction has two advantages over other tests generally used for the identification of the aerobic sporing bacilli. First, as it is much more selective, shown only by three closely related species, a positive yolk reaction will exclude many species otherwise similar. Secondly, the characteristic opaque zone around the colony enables a rapid recognition of *B. cereus* in a mixed culture. Confirmation of the usefulness of the test in the taxonomy of the *Bacillus* group depends on its application by many other workers to a greater number of strains.

Finally, as *B. cereus* and *B. mycoides* are among the most common laboratory contaminants, it would seem to be worth while to take precautions against contamination in the titration of certain toxins using the egg-yolk reaction, expecially when the reaction mixtures are incubated overnight.

We wish to express our thanks to Dr St John Brooks, formerly Curator of the National Collection of Type Cultures for his kindness in providing us with a large number of strains of aerobic sporing bacilli, also to Dr T. Gibson and Mrs A. C. Stirling, East of Scotland and Edinburgh College of Agriculture, for many standard strains of *B. cereus* and other species.

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EXPLANATION OF PLATE

Fig. 1. The yolk-broth reaction, after 24 hr. incubation: (1) *B. cereus*, active strain; (2) *B. mycoides*; (3) *B. cereus*, rather weak strain; (4) *B. anthracis*; (5) *B. subtilis*; (6) *B. megatherium*; (7) *B. licheniformis*; (8) uninoculated yolk broth.

Fig. 2. The isolation of *B. cereus* from soil by egg-yolk agar plate; note the four colonies with opaque zone.

Fig. 3. The egg-yolk agar plate test: (1) *B. cereus*, active strain; (2) *B. mycoides*; (3) *B. anthracis*; (4) *B. megatherium*; (5) *B. cereus*, rather weak strain; (6) *B. licheniformis*; (7) *B. subtilis*; (8) *B. anthracis*, strain 'vollum'; (9) *B. anthracis* 'H' variant of strain 'vollum'; (10) *B. anthracis* 'HM' variant of strain 'vollum'.

Fig. 4. Seven strains of *B. cereus* grown on yolk-agar plate (left) and blood-agar plate (right); note the correlation between yolk flocculating activity and haemolytic activity.

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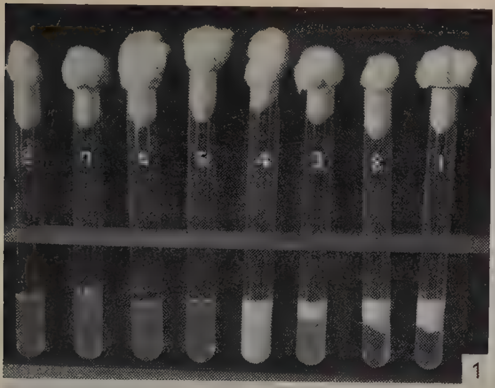
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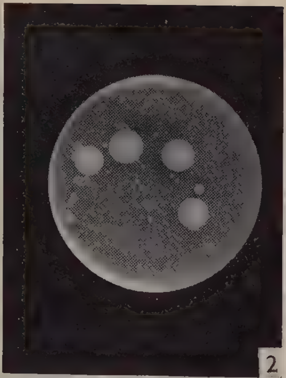
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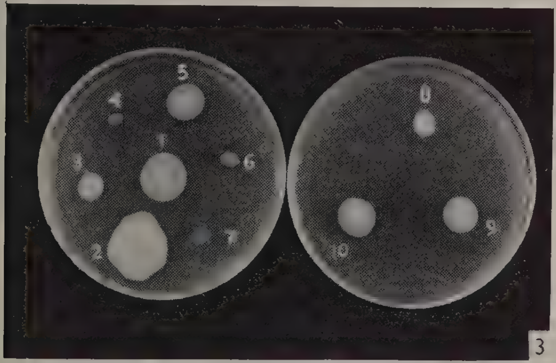

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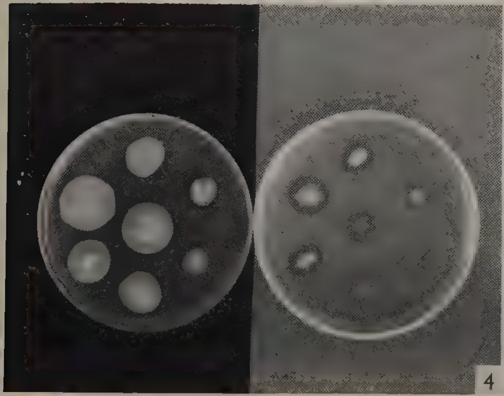
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Figs. 1–4

C. A. McGaughey and H. P. Chu—The egg-yolk reaction of aerobic sporing bacilli. Plate 1

Gladiolic Acid, an Antibiotic Substance Produced by *Penicillium gladioli* McCull. & Thom

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SUMMARY: Gladiolic acid is produced when *Penicillium gladioli* is grown on a wide range of culture media. The main factor influencing its production and accumulation is the pH drift of the medium, high yields being associated with a characteristic pH drift, consisting of an initial fall to about pH 4·0 followed by a steady but not too rapid rise. Continued low pH is unfavourable; too rapid a rise of pH is also unfavourable since gladiolic acid tends to disappear from the medium when pH 6·0 is reached, the disappearance being very rapid at pH 7·0 or above. The effects of variation of the initial pH of the medium, of glucose concentration, of variation of nitrogen source and of additions of certain organic acids are all explicable in terms of their effect on pH drift.

The antibiotic is best extracted from culture filtrates by treatment with activated charcoal after adjustment to pH 4·0, elution of the charcoal with ether, and recrystallization from water after evaporation of the ether. Yields of the order of 300 mg./l. are obtained.

Gladiolic acid is highly fungistatic if tested at low pH; the toxic effect is due to the undissociated molecules only and at pH 7·0, when dissociation is virtually complete, gladiolic acid is almost inactive. At pH 3·5, the least concentration inhibiting germination of fungus spores varies from 0·9 μg./ml. for *Fusarium graminearum* to 250 μg./ml. for *Trichoderma viride*. It is not highly antibacterial in broth, many organisms growing freely in the presence of 500 μg./ml. This low activity is thought to be due in part to the dissociation of gladiolic acid at pH 7·0 and in part to inactivation by certain broth constituents. This view is supported by the observation that bacterial cells suspended in gladiolic acid solutions (100 μg./ml.) in buffer at pH 4·0 are rapidly killed. This bactericidal effect occurs with both Gram-positive and Gram-negative organisms.

Gladiolic acid in solution is relatively stable in the range pH 3·0–8·0. In the presence of ammonium salts or certain amino-acids, notably *p*-aminobenzoic acid, it is rapidly inactivated, coloured complexes being formed. The reaction with ammonium salts is dependent on pH, not proceeding at pH 3·5 but proceeding rapidly at pH 7·0. The rapid disappearance of gladiolic acid in culture when the pH rises above 6·0 is possibly associated with this type of reaction.

Penicillium gladioli McCull. & Thom is found in many parts of the world as a weak parasite on *Gladiolus* corms in storage (Moore, 1939). It is uncertain whether infection of the corm takes place in the soil or at a later stage, but it seems certain that on some occasions infection takes place before the corms are lifted; this suggests that the fungus may be free-living in the soil, though no record exists of its direct isolation from soil.

In a preliminary communication (Brian, Curtis, Grove, Hemming & McGowan, 1946) strains of *P. gladioli* were shown to produce a strongly antifungal and weakly antibacterial substance, gladiolic acid, which appeared to be a methoxy methyl 2-carboxyphenyl glyoxal ($C_{11}H_{10}O_5$). The present communication is confined to a more detailed study of the conditions of production of gladiolic acid and of its biological properties; its chemistry will be dealt with in a separate publication.

Methods

Assays of fungistatic and bacteriostatic activity. For routine assays of fungistatic activity a spore germination test has been used, with conidia of *Botrytis allii* Munn. The results are expressed in arbitrary B.A. units/ml.; a B.A. unit is that quantity of antibiotic which, dissolved in a stated quantity of Weindling medium, reduces germination of *B. allii* spores to 2 % or less. Details of this technique have been previously described (Brian & Hemming, 1945). Unless otherwise stated, all solutions were adjusted to pH 3·5 before assay of fungistatic activity. Assays of bacteriostatic activity were made by serial dilutions in broth. Other special methods, where used, are described later.

Methods of culture. In experiments which did not involve extraction of gladiolic acid, cultures were grown on 250 ml. lots of medium in 'Glaxo' culture vessels (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). Samples of the underlying medium were withdrawn periodically for assay, under sterile conditions. In general, six cultures were set up on each medium in an experiment, the samples for each vessel in each set of six being bulked for assay. For bulk production, earthenware culture vessels, each holding 0·5–1·0 l., were used.

A crude grade of glucose was used in all media unless otherwise stated. All cultures were grown at 25°.

Production of spores for inoculum. Sporulation of *P. gladioli* is closely dependent on temperature. McCulloch & Thom (1928) record that at 20° few conidiophores are produced, though sclerotia are formed abundantly, whereas at 15° conidiophores and conidia are produced freely, with few sclerotia. Gladiolic acid is produced by both of the two strains of *P. gladioli* examined: no. 59 (N.C.T.C. 3994), isolated by F. T. Brooks in 1931, can be characterized as a conidial strain, producing conidia abundantly at room temperature (*c.* 15–20°), with few sclerotia; no. 206, isolated from a *Gladiolus* corm in 1944, can be characterized as a sclerotial strain, producing sclerotia abundantly, with very few conidiophores, even at room temperature. Since easy production of spores for inoculum was a matter of importance, strain no. 59 was used in all the experiments now described. It was found best to grow the mould on Czapek-Dox agar in flat medicine bottles; these are incubated for 3–4 days at 25° and the bottles are then removed from the incubator and kept at room temperature. Under these conditions conidia are produced abundantly, though few are produced if the cultures are incubated at 25° throughout. Conidia were removed from the cultures by adding a little sterile water to the bottle and gently rubbing the agar with a sterile glass rod.

Relation between medium and development of fungistatic activity

Preliminary experiments indicated that Raulin-Thom medium was more effective than Czapek-Dox, somewhat higher titres being attained and maintained for several days, whereas on Czapek-Dox a sharp decline followed the development of peak activity. Accordingly, production of gladiolic acid in a medium of the Raulin-Thom type was studied, and in later stages, in a simplified medium. The gladiolic acid was not extracted, but was assayed by the *B. allii* spore-germination test. It was assumed that all activity was due to gladiolic acid. Our experience in extraction experiments has justified this assumption.

The variables studied included initial pH of the medium, concentration of carbon source (glucose), type of nitrogen source, and the effect of additions of malic acid to ammonia-nitrogen media. All the experimental data presented below indicate that these factors mainly influence the pH drift of the medium during growth of the mould.

Table 1. *Effect of initial* pH *of Raulin-Thom medium* (7·5 % (*w*/*v*) *glucose*) *on fungistatic activity* (B.A. *units/ml.*) *of* Penicillium gladioli *cultures*

Initial pH	Days growth					Mean final dry wt. of mycelium (g./culture)
	4	8	11	15	21	
	B.A. units/ml.					
3·2	—	—	—	—	—	2·7
4·2	—	12	32	48	48	3·0
5·0	8	64	24	24	4	3·3
6·1	6	96	32	32	6	3·1
6·8	6	96	24	24	4	3·1

Initial pH *of Raulin-Thom medium.* Raulin-Thom medium was prepared, according to the formula given by Brian, Curtis & Hemming (1946), with initial pH ranging from 3·2 to 6·8. The glucose and salts were made up in separate solutions, the pH of the salt solution being adjusted with HCl or KOH before autoclaving; and the two sterile solutions mixed under sterile conditions. This procedure avoids the considerable breakdown of glucose that occurs if autoclaved in the complete medium at high pH. Results of periodical assays and final dry weights of the mycelium from *P. gladioli* cultures on these media are recorded in Table 1.

It will be seen that the low initial pH does not favour the production of fungistatic substances, none developing in the medium initially adjusted to pH 3·2 and some developing only slowly in the medium initially adjusted to pH 4·2. Growth and development of the fungus were also affected; on the pH 3·2 medium the mycelial felt produced was never completely confluent and few, if any, conidia were formed. At pH 4·2 growth was confluent, but sporulation was reduced. At higher pH values thick, vigorously sporing felts were rapidly produced. The final dry weight of mycelium (i.e. after 21 days' growth) was not very markedly affected by initial pH, though the same general trend was observed; the failure to produce gladiolic acid in media of low pH cannot be attributed to reduction in the growth rate of the mould. Study of the pH drift (Fig. 1) reveals a significant difference between the media rapidly producing high concentrations of gladiolic acid and the two poorer ones. The pH 3·2 medium shows an initial fall to pH 2·0 followed by a slow rise to pH 2·6. The pH 4·2 medium shows an initial fall to pH 3·0 followed by a rise to pH 5·1 and the fungistatic activity develops during the phase of rising pH. The remaining three media (pH 5·0, 6·1, 6·8) show similar pH drifts; there is an initial fall to near pH 3·0, followed by a rapid rise to pH 6·0–6·5 after which a fairly steady level is maintained. Fungistatic activity develops rapidly during the phase of rising pH, but then falls. The significance of these relations becomes clearer after consideration of further experiments.

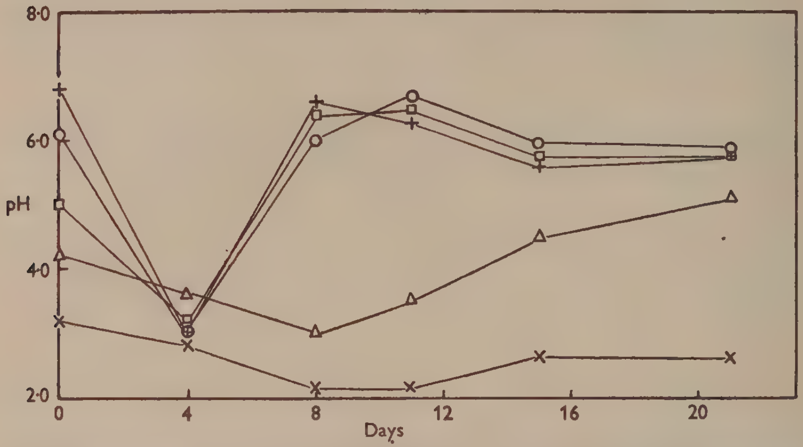

Fig. 1. Effect of initial pH of Raulin-Thom medium on pH drift in cultures of *Penicillium gladioli*. (×, pH 3·2; △, pH 4·2; □, pH 5·0; ○, pH 6·1; +, pH 6·8.)

Table 2. *Effect of glucose content of Raulin-Thom medium* (pH 5·5) *on fungistatic activity* (B.A. *units/ml.*) *of* Penicillium gladioli *cultures*

Dextrose (% w/v)	Days growth B.A. units/ml. 4	6	9	12	16	20	Mean final dry wt. of mycelium (g./culture)
1·0	—	—	—	—	—	—	2·1
2·5	16	2	—	—	—	—	3·4
5·0	8	48	32	16	2	—	3·1
7·5	6	16	64	48	16	8	4·8
10·0	6	12	32	96	48	24	4·7
15·0	4	8	12	24	96	96	4·6

Glucose concentration in Raulin-Thom medium. P. gladioli was grown on Raulin-Thom medium made up, at pH 5·5, with glucose concentrations ranging from 1·0 to 15·0 % (w/v). Results of assays, pH drift and mycelial weights are recorded in Table 2 and Figs. 2 and 3. This experiment is particularly illuminating. The following points should be noted.

(*a*) The typical pH drift is an initial fall followed by a rise, the rate of rise in pH being inversely proportional to the glucose concentration. Thus, for example, the medium with 15·0 % falls to pH 3·5 in 4 days and thereafter rises slowly to pH 5·8 after 20 days; the medium with 2·5 % glucose falls to pH 4·4 in 4 days and thereafter rises rapidly, reaching pH 7·0 in a further 2 days,

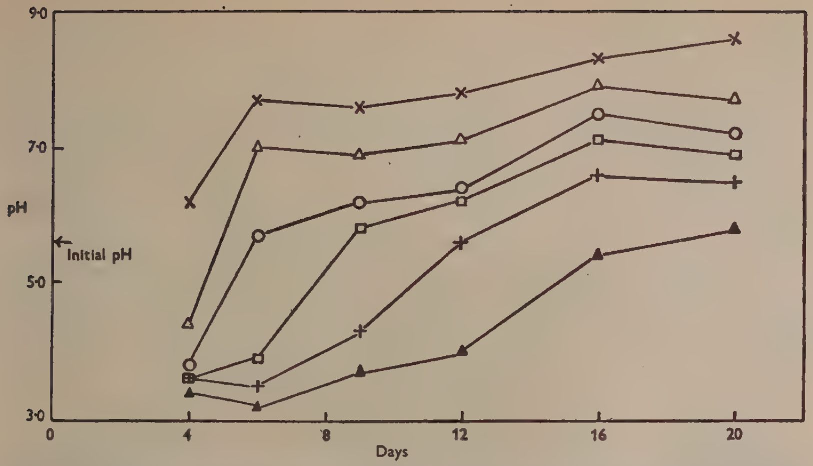

Fig. 2. Relation between glucose concentration and pH drift in cultures of *Penicillium gladioli* on Raulin-Thom medium. (×, 1·0 % glucose; △, 2·5 %; ○, 5·0 %; □, 7·5 %; +, 10·0 %; ▲, 15·0 %.)

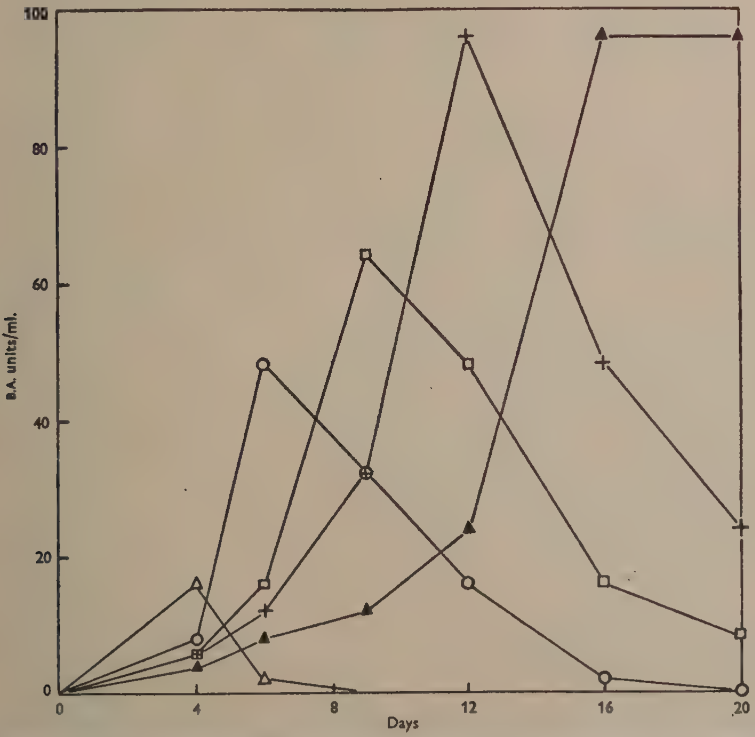

Fig. 3. Relation between glucose concentration and development of fungistatic activity in cultures of *Penicillium gladioli* on Raulin-Thom medium. (△, 2·5 % glucose; ○, 5·0 %; □, 7·5 %; +, 10·0 %, ▲, 15·0 %.)

after which the change in pH is more gradual. The medium with only 1·0 % glucose is anomalous to the extent that no initial fall in pH was recorded, though this might have been detected if the medium had been tested earlier.

(*b*) Initially, the rate of development of fungistatic activity (neglecting for the moment the medium with 1·0 % glucose) is inversely related to the glucose concentration.

(*c*) The peak of fungistatic activity is directly related to the glucose concentration, a slow initial rise in assay being correlated with a higher peak assay.

(*d*) Fungistatic activity, after reaching a maximum, falls off in all media save that with 15·0 % glucose. The fall in activity in each case coincides with the development in the medium of a pH above 6·0. The absence of a fall in activity in the medium with 15·0 % glucose is consistent with the fact that at the end of the experiment the critical pH 6·0 had not been reached.

(*e*) Although with increasing glucose concentration the weight of mycelium formed increases, the difference in mass of mycelium cannot be held to account for the differences in gladiolic acid assay.

These experiments on the effect of glucose concentration and initial pH strongly suggest that an upward trend of pH in the medium favours gladiolic acid production or accumulation, but that once pH 6·0 is reached the gladiolic acid is progressively destroyed. This view was confirmed by an experiment using different carbon sources. The fungus was unable to utilize lactose; little growth took place and no gladiolic acid was produced. Starch, dextrin, sucrose, glucose and glycerol were all utilized; the pH drift with glycerol and sucrose was much more gradual than with glucose and higher assays were maintained much longer with these two materials. In each case activity began to fall when pH 6·0 was reached.

Nitrogen source and organic acid supplement. In an experiment with a number of different nitrogen sources a simplified medium (Brian, Curtis & Hemming, 1947) was used. The nitrogen sources were potassium nitrate, ammonium nitrate, ammonium sulphate and ammonium tartrate. Experience with other fungi suggested that at times the whole course of metabolism may differ if ammonia nitrogen is substituted for nitrate nitrogen. There is also evidence (Brian *et al.* 1947) that utilization of ammonia nitrogen may be facilitated by the presence of certain organic acids and hence ammonium tartrate was compared with ammonium sulphate; supplements of malic acid (0·5 % w/v) to ammonium sulphate media were tested for similar reasons.

The various nitrogen sources were added to give nitrogen equivalent to 0·23 % potassium nitrate, at two glucose levels (2·5 and 10·0 %), the media being adjusted to pH 5·5. *P. gladioli* was grown on these media in the usual way. It is quite clear from these data (Table 3 and Fig. 4) that the form of nitrogen supplied is of little importance except in so far as it affects the drift of pH in the medium. Ammonium sulphate alone failed to give high assays with either low or high glucose concentrations. This is probably explained by the uniform low pH of ammonium sulphate media, previously shown to be unfavourable to gladiolic acid production; in the series with high sugar concentration growth in ammonium sulphate media was relatively reduced, but not to a sufficient extent to explain the low assays. Addition of malate to ammonium sulphate media led to an upward pH drift and correspondingly higher assays. With all the media the assay rose with a rising pH drift in the medium, falling when a pH above 6·0 was reached. The results are of interest, too, in showing that a low glucose concentration (2·5 %) is not intrinsically unfavourable; in a medium with ammonium tartrate as nitrogen source, in which the pH does not rise too rapidly, quite high assays were recorded, though activity fell rapidly as soon as pH 6·0 was reached. It remains generally true, therefore, that to maintain high assays a high sugar concentration in the medium is necessary.

Table 3. *Effect of nitrogen source, at two glucose concentrations, on development of fungistatic activity* (B.A. *units/ml.*) *in* Penicillium gladioli *cultures*

Nitrogen sources in these media are as follows: N, potassium nitrate; AN, ammonium nitrate; AS, ammonium sulphate; AS + M, ammonium sulphate + malate; A, ammonium tartrate.

Medium	Glucose (% w/v)	Days' growth							Mean final dry wt. of mycelium (g./culture)
		4	6	8	11	15	18	22	
		B.A. units/ml.							
N	2·5	6	4	2	—	—	—	—	1·3
AN	2·5	4	32	16	—	—	—	—	1·4
AS	2·5	4	4	2	2	2	—	—	1·2
AS + M	2·5	6	8	2	—	—	—	—	1·1
A	2·5	4	64	32	12	—	—	—	1·2
N	10·0	6	12	24	64	32	48	96	3·3
AN	10·0	4	16	16	32	32	32	48	3·9
AS	10·0	4	6	2	2	2	2	2	2·2
AS + M	10·0	4	32	32	64	96	32	24	3·2
A	10·0	2	8	12	16	24	32	48	5·8

The effect of malic acid supplements in making ammonium sulphate media favourable for gladiolic acid production and accumulation was also studied. To an ammonium sulphate medium malic acid supplements were added in the range 0·05–1·0 % (w/v), all media being adjusted to pH 5·5 with KOH. From the results of assays and pH drifts in *P. gladioli* cultures on these media (Table 4 and Fig. 5) it will be seen that the medium without supplement shows a slow fall in pH and that as the concentration of added malic acid is increased the slow fall was replaced by a rapid rise. The assays show similar relations to pH as were observed in previous experiments. The optimum medium is that with 0·25 % malic acid, which produced the gradual rise in pH previously found to be favourable.

It has been shown (Brian *et al.* 1947) that malate supplements to an ammonium sulphate medium greatly stimulate growth of *Metarrhizium glutinosum* Pope (= *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr.) and production of the antibiotic by that mould. The action of malate was attributed mainly to its effect on ammonia assimilation, and its action on the pH drift in the medium was regarded as secondary. Growth on unsupplemented ammonium sulphate media was negligible and small concentrations of malate, or certain other 2- to 5-carbon acids, greatly stimulated growth of *M. verrucaria* under conditions where the pH drift was not significantly affected. The behaviour of *P. gladioli* is not considered to be parallel, since growth, which is quite vigorous on unsupplemented ammonium sulphate media, is not affected; malate only affects production of gladiolic acid considerably in concentrations markedly affecting the pH drift and the effect of malate can be explained entirely by its effect on pH drift.

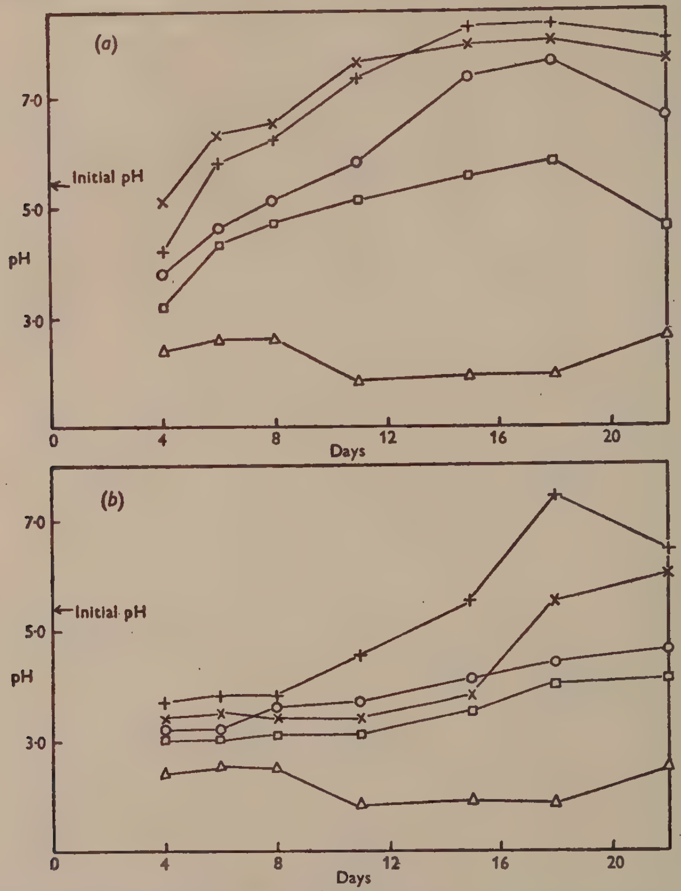

Fig. 4. Effect of nitrogen source on pH drift in cultures of *Penicillium gladioli* (*a*) 2·5 % glucose, (*b*) 10·0 % glucose. (×, KNO_3; ○, NH_4NO_3; △, $(NH_4)_2SO_4$; □, ammonium tartrate; +, $(NH_4)_2SO_4$+malate.)

We conclude that the main factor influencing gladiolic acid production and accumulation in cultures of *P. gladioli* is the pH drift of the medium. Gladiolic acid accumulation is not favoured by a continued low level of pH; it is favoured by an upward drift of pH, but once the pH drift of the medium reaches a level above pH 6·0 the gladiolic acid is progressively destroyed. The significance of these relations is discussed below in connexion with the toxicity and stability of gladiolic acid.

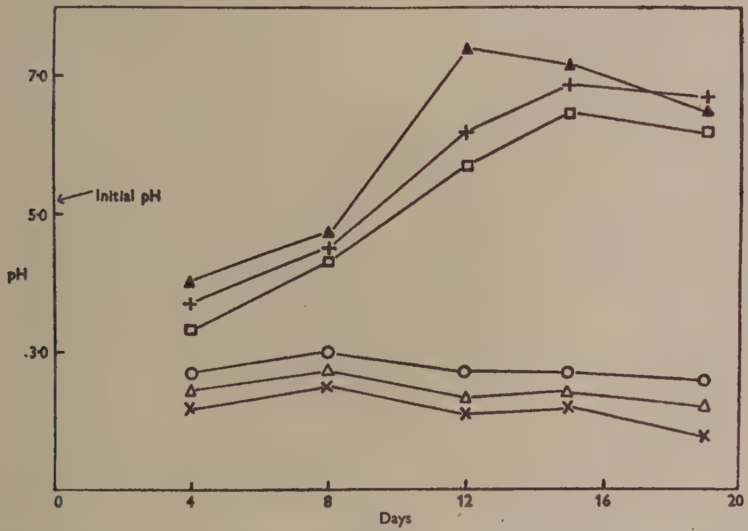

Fig. 5. Effect of malate supplements on pH drift in cultures of *Penicillium gladioli* on an ammonium sulphate medium. (×, no malate; △, 0·05 % malate; ○, 0·01 %; □, 0·25 %; +, 0·5 %; ▲, 1·0 %.)

Table 4. *Effect of malate supplements in an ammonium sulphate medium on development of fungistatic activity* (B.A. *units/ml.*) *in* Penicillium gladioli *cultures*

Malic acid (% w/v)	Days' growth					Mean final dry wt. of mycelium (g./culture)
	4	8	12	15	19	
	B.A. units/ml.					
—	4	2	2	—	2	2·0
0·05	8	8	12	8	12	2·0
0·1	24	24	24	16	24	2·0
0·25	16	192	192	96	64	2·1
0·5	24	96	96	48	24	2·2
1·0	12	96	82	24	12	2·0

Extraction and purification of gladiolic acid

Samples of a filtrate from Raulin-Thom cultures, of final pH 4·4 and activity of 48 B.A. units/ml. were extracted with *n*-butanol (2 × 0·2 vol.), chloroform (3 × 0·1 vol.), ether (2 × 0·2 vol.) and light petroleum (b.p. 40 – 60°) (3 × 0·1 vol.). Of these solvents chloroform was the most effective, but not more than 50 % of the activity was removed. Treatment with activated charcoal (British Drug

Houses Ltd.) at 5 g./l. removed all activity. The active material could be recovered from the charcoal by elution with acetone, ether, methanol, ethanol or chloroform. Acetone was the most effective in that relatively small volumes were needed, but it also removed considerable quantities of pigment which made further purification more difficult. Ether was chosen as eluent; it was almost as effective as acetone and did not extract appreciable quantities of pigment. Elution was most satisfactory in a Gallenkamp Universal Extractor, in which it was consistently more rapid than in the usual Soxhlet extractor (cf. the elution of glutinosin from charcoal; Brian, *et al.* 1947). On evaporation of the ethereal eluate a yellow pasty mass was obtained, from which pure gladiolic acid, in the form of long, colourless, silky needles, was obtained by several recrystallizations from water.

The pH of extraction is important. A Raulin-Thom culture filtrate, assaying at 32 B.A. units/ml., was divided and samples adjusted to pH 2·0, 3·0, 4·0 and 5·4 before treatment with charcoal. Yields were respectively 0, 133, 154 and 86 mg./l. Better extraction at pH 3·0 or 4·0 than at 5·4 could be expected, but the failure of extraction at pH 2·0 cannot at present be explained. For routine production purposes all culture filtrates are now adjusted to pH 4·0 before extraction.

With Raulin-Thom medium (7·5 % glucose) yields ranged from 150 to 350 mg./l.; with Czapek-Dox (7·5 % glucose) from 150 to 200 mg./l.; with medium AN (7·5 % glucose) from 100 to 250 mg./l. Yields of the order of 300 mg./l. can now be consistently obtained on Raulin-Thom medium (7·5 % glucose) of initial pH 5·0.

Biological activity of gladiolic acid

Fungistatic activity. The least concentration of gladiolic acid required to produce 95–100 % inhibition of germination of spores of a number of fungi in Czapek-Dox medium at pH 3·5 is given in Table 5. There is a wide difference in susceptibility among the fungi tested, the lethal dose varying from 0·9 μg./ml. for *Fusarium graminearum* to 250·0 μg./ml. for *Trichoderma viride.*

Table 5. *Fungistatic activity of gladiolic acid at* pH 3·5

Fungus	Least inhibiting concentration (μg./ml.)	Fungus	Least inhibiting concentration (μg./ml.)
Absidia glauca Hagem	1·9	*Penicillium digitatum* Sacc.	15·6
Aspergillus flavus Link	125·0	*P. expansum* Link	3·9
A. niger van Tiegh.	125·0	*P. gladioli* McCull. & Thom	7·8
Botrytis allii Munn.	7·8	*P. janczewskii* Zal.	1·9
Byssochlamys fulva Olliver & Smith	1·9	*P. notatum* Westling	3·9
		Stemphylium sp.	62·5
Cephalosporium longisporum Petch	15·6	*Thamnidium elegans* Link	62·5
Fusarium caeruleum (Lib.) Sacc.	3·9	*Trichoderma viride* Pers. ex Fries	250·0
F. graminearum Schwabe	0·9		
Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fries	62·5	*Trichothecium roseum* Link	250·0
Mucor erectus Bain.	7·8	*Verticillium alboatrum* Reinke & Berth.	7·8

The fungistatic activity of gladiolic acid varies with the pH of the solution, as is usually the case with weak acids. In Table 7, the fungistatic activity of a 0·2% solution of gladiolic acid at pH 3·0 is 512 B.A. units/ml., falling to only 2 B.A. units/ml. at pH 7·0. These results are best explained on the assumption that it is only the lipid-soluble, undissociated gladiolic acid molecules which penetrate the plasma membrane of the spore.

It will be noted that gladiolic acid is quite toxic to *P. gladioli* at pH 3·5. This suggests an explanation of the increased gladiolic acid production in cultures with a rising pH; if the pH remained level near pH 4·0, a concentration of gladiolic acid toxic to *P. gladioli* (*c.* 10 mg./l.) would very soon be reached, but with a rising pH increased dissociation of gladiolic acid molecules allows the accumulation of much higher concentrations (greater than 300 mg./l.) without any undue autotoxic effect.

Antibacterial activity. When included in broth at pH 7·0 gladiolic acid exerts little bacteriostatic effect. Growth of *Staphylococcus aureus* (two strains), *Salmonella typhi*, *Bacterium lactis aerogenes*, *B. subtilis*, *B. brevis*, *Escherichia coli* and *Micrococcus lysodeikticus* were not inhibited by 500μg./ml. gladiolic acid. The other strains of *S. aureus* were inhibited at 250μg./ml.

Nevertheless, under certain conditions, gladiolic acid is markedly bactericidal. *S. aureus* (N.R.R.L. 313), *Salmonella typhi* (N.C.T.C. 786) and *Esch. coli* (N.C.T.C. 419) were suspended, at 20°, in broth adjusted to pH 4·0 and 7·0 and in citric acid-phosphate buffers at pH 4·0 and 7·0, containing in each case gladiolic acid at 10 and 100μg./ml. Loopfuls were taken out after 6, 12, 18, 24, 30, 45 and 60 min. and streaked on nutrient agar. There was no bactericidal effect with either concentration of gladiolic acid in broth at either pH. The times taken for 100μg./ml. gladiolic acid to produce a complete, or virtually complete, kill in buffer at pH 4·0 were *Staph. aureus* 18 min., *Salmonella typhi* 12 min., *Esch. coli* 18 min. In buffer at pH 7·0 the corresponding times were: *Staph. aureus* >60 min., *S. typhi* 18 min., *Esch. coli* 45 min. Gladiolic acid at 10μg./ml. in buffer at pH 4·0 caused some kill of *S. typhi* in 60 min. but did not affect the other organisms; at pH 7·0 this concentration had no effect on any of the organisms. Thus the antibacterial effect of gladiolic acid is related to pH, as was the antifungal effect, and its antibacterial activity is reduced by some constituent of broth (see below).

Stability of gladiolic acid solutions in relation to pH

Gladiolic acid solutions (0·2%) in Weindling and Czapek-Dox media at pH 3·0, 5·0, 7·0 and 8·0 were assayed after various heat treatments. All solutions were readjusted to pH 3·5 before assay. The results presented in Table 6 indicate (*a*) that in either medium at pH 3·0 gladiolic acid is relatively stable, (*b*) that in Weindling medium at pH 5·0 or above gladiolic acid is rapidly inactivated even in the cold, but (*c*) that in Czapek-Dox it is much more stable and even at pH 8·0 severe heat treatment is necessary to produce any considerable loss in activity.

In the Weindling medium loss in activity is associated with the formation of a green precipitate; it was recorded previously (Brian *et al.* 1946) that gladiolic acid gives a green colour when treated with ammonia, and it therefore seemed probable that the inactivation of gladiolic acid in Weindling was due to reaction with ammonium tartrate. Czapek-Dox contains no ammonium salts, nitrogen being supplied as nitrate.

Table 6. *Effect of heat treatments on fungistatic activity of gladiolic acid solutions*

pH of solution	No treatment	Boiled under reflux (min.)			Autoclaved at 120° (min.)			
		5	15	60	10	20	30	60
	B.A. units/ml.							
	(*a*) In Czapek-Dox medium							
3·0	255	192	192	256	256	192	128	128
5·0	256	192	256	192	192	192	128	32
7·0	256	192	128	96	64	64	4	4
8·0	256	64	64	64	32	32	2	2
	(*b*) In Weindling medium							
3·0	192	192	128	192	192	192	128	128
5·0	8	6	6	8	2	4	4	4
7·0	—	—	—	—	—	—	—	—
8·0	—	—	—	—	—	—	—	—

Table 7. *Activity of* 0·2 % *gladiolic acid solutions in Czapek-Dox* (*a*) *assayed after readjustment to* pH 3·5, (*b*) *assayed at* pH *of solution*

pH of solution	Days stored at 25°				
	0	1	2	3	8
	B.A. units/ml.				
	(*a*) Assayed at pH 3·5				
3·0	256	192	192	256	192
4·0	256	192	128	192	256
5·0	256	128	192	128	192
6·0	256	192	192	192	192
7·0	256	128	192	256	192
8·0	256	128	128	192	192
	(*b*) Assayed at pH of solution				
3·0	512	256	512	512	512
4·0	96	96	128	128	96
5·0	16	16	16	16	16
6·0	2	2	2	2	2
7·0	2	2	2	2	2
8·0	—	—	2	2	—

At physiological temperatures gladiolic acid is very stable in Czapek-Dox between pH 3·0 and 8·0 (Table 7). Similar experiments indicated that at pH 3·5 or below it is quite stable in Weindling medium. Table 7 also shows that the activity of gladiolic acid is dependent on the pH of the solution.

Inactivation of gladiolic acid by ammonium salts and amino-acids

Ammonium salts. The suspected role of ammonium salts in the inactivation of gladiolic acid in Weindling medium was confirmed and the inactivation studied by periodical assays of mixtures of gladiolic acid and ammonium chloride in McIlvaine's citric acid-phosphate buffer (Table 8). It will be seen that (*a*) the inactivation is dependent on pH, proceeding at pH 5·0 and even more rapidly at pH 7·0 but not at pH 3·3, (*b*) the process is relatively slow at the temperature chosen (25°), and (*c*) even at the most favourable pH level an excess of ammonium chloride molecules is needed to produce complete inactivation, though activity drops to 25 % of the original where ammonium chloride and gladiolic acid are present in equimolecular proportions. In all cases where appreciable inactivation took place yellow or green colours developed in the solutions.

Table 8. *Fungistatic activity of mixtures of gladiolic acid and ammonium chloride in McIlvaine's buffer in relation to* pH

Composition of mixture assayed			Days stored at 25°		
			0	1	4
			BA.. units/ml.		
Gladiolic acid (M)	NH_4Cl (M)	pH			
0·001	—	3·3	24	24	32
—	0·1	3·3	—	—	—
0·001	0·1	3·3	24	32	32
0·001	0·01	3·3	32	32	32
0·001	0·001	3·3	24	32	32
0·001	—	5·0	32	24	24
—	0·1	5·0	—	—	—
0·001	0·1	5·0	16	2	—
0·001	0·01	5·0	16	8	8
0·001	0·001	5·0	32	24	16
0·001	—	7·0	24	24	24
—	0·1	7·0	—	—	—
0·001	0·1	7·0	16	—	—
0·001	0·01	7·0	24	—	—
0·001	0·001	7·0	16	12	6

Amino-acids. In their studies on glyoxalase, Dakin & Dudley (1913, 1914) recorded a reaction between phenyl glyoxal and ammonia, giving coloured products and between phenyl glyoxal and certain amino-acids, notably histidine, arginine, ornithine and lysine, to give sparingly soluble yellow substances. Accordingly, in view of the structural relationship between gladiolic acid and phenyl glyoxal, the activity of gladiolic acid in the presence of certain amino-acids has been studied (Table 9). Several amino-acids inactivated gladiolic acid, the most effective, in descending order, being *p*-aminobenzoic acid, histidine, tryptophan, arginine and glycine. In all cases where marked inactivation took place yellow or green colours were produced. *p*-Aminobenzoic acid was outstanding, producing immediate and complete inactivation when mixed in equimolecular proportions with gladiolic acid; it did not completely inactivate when present in less than equimolecular proportions. *p*-Aminobenzenesulphonamide was equally effective; anthranilic (*o*-aminobenzoic acid) was somewhat less effective and the results were complicated by the marked fungistatic activity of this compound.

Table 9. *Fungistatic activity of mixtures of gladiolic and various amino-acids in Czapek-Dox* (pH 3·5), *assayed immediately and after* 24 *hr. storage at* 25°

The 0·001 M gladiolic acid alone showed an activity of 16 B.A. units/ml.; none of the amino-acids alone showed fungistatic activity at 0·01 M, neither did they stimulate.

Animo-acid	0·001 M gladiolic acid + 0·01 M amino-acid		0·001 M gladiolic acid + 0·001 M amino-acid	
	0 hr.	24 hr.	0 hr.	24 hr.
	B.A. units/ml.		B.A. units/ml.	
DL-Alanine	16	16	16	12
p-Aminobenzoic acid	—	—	—	—
L-Arginine hydrochloride	8	2	16	12
L-Asparagine	16	16	24	16
L-Aspartic acid	12	6	24	8
L-Cystine	12	12	16	16
L-Glutamic acid	12	4	16	16
Glycine	8	4	12	12
L-Histidine hydrochloride	—	—	12	4
L-Leucine	16	16	16	24
L-Phenylalanine	16	16	16	16
L-Proline	16	8	16	16
L-Tryptophan	8	—	12	6
L-Tyrosine	12	8	16	16

Discussion

The inactivation of gladiolic acid by ammonium salts and amino-acids may be of significance in explaining the disappearance of gladiolic acid from cultures of *P. gladioli* when the pH rises above 6·0 and the mechanism of the antifungal action of gladiolic acid.

Cultures of *P. gladioli* produce gladiolic acid most abundantly in circumstances where, after an initial fall, there is a rising trend in pH, but that after pH 6·0 or thereabouts is reached gladiolic acid rapidly disappears. As has been shown, gladiolic acid is intrinsically stable under such conditions, but also does react with ammonium salts at that pH, forming an insoluble inactive compound. Ammonium salts are normally present in cultures after several days' incubation, even if nitrogen is originally supplied as nitrate, so there is every opportunity for this kind of reaction to occur; in fact dark green precipitates, similar in appearance to those produced by reaction of ammonium salts with gladiolic acid, were observed in cultures, particularly frequently when nitrogen was supplied as ammonium nitrate.

The inactivation of gladiolic acid by certain amino-acids, notably *p*-aminobenzoic acid, histidine and tryptophan, suggests that its mode of action may be concerned with immobilization of such amino-acids in the fungal cell. This possibility will be investigated. It is not suggested that the inactivation of gladiolic acids by amino-acids is in any strict sense specific; it is highly probable that many aromatic amines would have a similar effect.

We wish to thank Miss Valerie Spence, Mr G. W. Elson and Mr C. H. Unwin for much valuable assistance in this investigation.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its meeting in the Lecture Theatre of the Departments of Biochemistry and Physiology, the University Museum, Oxford which consisted of (a) *a Symposium on 'Amino-acids in the Economy of Micro-organisms' on Friday,* 19 *September* 1947, *and* (b) *reading of original papers on Saturday,* 20 *September* 1947. *The following communications were made:*

COMMUNICATIONS

AMINO-ACIDS IN THE ECONOMY OF MICRO-ORGANISMS

Introductory Survey. By D. D. Woods

The main object of this Symposium is to discuss in a broad way the part played by amino-acids in the life of the cell rather than to describe in full detail the many interesting chemical reactions involved. For convenience only the main functions of amino-acids may be divided into two main types: (*a*) processes involving direct utilization for the synthesis of new cell material, (*b*) processes involving breakdown of amino-acids.

Anabolic processes

The high rate of reproduction of many micro-organisms implies rapid and massive synthesis of new cell material. Some of this is protein, which is in turn built up from amino-acids. We shall consider first therefore the source of the amino-acids. They must either (*a*) be provided in the growth medium as such or as compounds from which the organism can liberate the amino-acids (e.g. peptides, protein), or (*b*) be synthesized by the organisms themselves from simpler compounds. The ability to synthesize the amino-acids required varies greatly from one group of micro-organisms to another and even within the same species, and these substances often appear as essential growth factors. Practical use is made of this in the microbiological assay methods which have been developed for the quantitative determination of amino-acids. When amino-acids have to be provided preformed, the mechanism of their assimilation by the cell becomes of importance.

When a number of different species, or a number of mutants (natural or induced) of the same species, require a given amino-acid preformed, it is possible that the chain of reactions by which the synthesis is achieved has failed at different stages with the different organisms. Important information as to the detailed chemistry of the synthesis of several amino-acids has followed from such studies, which have further significance in that they have also led to progress in defining genetic changes in biochemical terms.

In so far as studied bacteria seem to be capable of synthesizing protein if the amino-acids are available or can be synthesized. More elaborate molecules such as peptides may be essential for certain Protozoa. The growth of certain haemolytic streptococci and lactobacilli is, however, stimulated greatly by 'strepogenin' which appears to be a peptide. This is a new and promising field in growth-factor research.

Catabolic processes

The aerobic or anaerobic decomposition of amino-acids may have several functions in cell economy. Among such functions are (*a*) the provision of energy for coupling to the synthetic processes of the cell, (*b*) the production of nitrogen-free intermediates for use in the synthesis of non-nitrogenous cell constituents, (*c*) the production (by deamination) of ammonia which may be used as primary nitrogen source for the synthesis of other nitrogenous compounds, (*d*) possibly as a mechanism for controlling hydrogen-ion concentration in the environment. Some of the enzymes concerned in amino-acid decomposition are adaptive in nature.

Interrelationships with the economy of other organisms

(1) *Other micro-organisms.* Here amino-acids may play a part in widely differing ways. First, life in mixed culture may depend on the synthesis by some organisms of the amino-acids required as growth factors by others. Secondly, amino-acids or derivatives are involved in the constitution of several antibiotics (e.g. gramicidin, penicillin). Thirdly, amino-acids are implicated in the absorption of bacteriophage by the host bacterium.

(2) *With higher organisms.* Some bacterial toxins (e.g. *Cl. botulinum*) have been shown to be proteins, though so far nothing abnormal has been detected in their amino-acid composition. In the field of immunology, a notable example is the capsular antigen of *B. anthracis*; here specificity is conferred by the capsular polypeptide, which is built up entirely from *d*-glutamic acid units.

Biosynthesis of Amino-acids. By ANDRÉ LWOFF

This summary is restricted to some points concerning amino-acid synthesis.

Synthesis of the amino group

(*a*) Some free-living and some symbiotic bacteria are able to reduce elementary nitrogen. It is generally assumed that one of the primary products is nitrogen hydrate which may be oxided to hyponitrous acid or be reduced to hydroxylamine. When molecular nitrogen enriched with N^{15} is furnished to *Azotobacter vinelandi*, the highest level of N^{15} in the amino fraction appears in glutamic acid fraction, the next in aspartic acid fraction.

(*b*) Many bacteria are able to utilize NO_3^- as nitrogen source, that is to say, to reduce NO_3^- to NH_3. The second step is the conversion of nitrous acid into ammonia, with perhaps the formation of hydroxylamine as intermediary step.

(*c*) Many micro-organisms are unable to utilize N_2, NO_3^- or NO_2^- as nitrogen source and need NH_3 as nitrogen source.

Synthesis of ternary part

Photosynthetic and chemosynthetic bacteria are able to reduce CO_2 to (CH_2O). Some of the building blocks of amino-acids are synthesized directly from CO_2 and (CH_2O), some others presumably from the degradation product of intermediary carbohydrates and specially from pyruvic acid. No data are available on the relative importance of the two phenomena during bacterial growth. Pyruvic acid may also be formed by a reaction between formate and acetylphosphate.

The fact that pyridoxal phosphate is the coenzyme of amino-acid decarboxylases raises the question of reversibility of the decarboxylation and of the possible role of vitamin B_6 in the carboxylation processes and in some amino-acid synthesis. The importance of CO_2 for bacterial growth is well known. Low CO_2 tension decreases the growth rate. This decrease is compensated by C_4 or C_5 dicarboxylic acids. They do not act in the absence of CO_2; but yeast extract and bacterial lysates are potent. Thus CO_2 plays a fundamental part in bacterial growth for the synthesis of essential heterocarboxylic metabolites.

The decarboxylation of pyruvate into phosphoacetate requires inorganic phosphate and diphosphothiamine. The importance of phosphorylation in the reverse process seems certain. It seems also probable that phosphorylation is necessary for the carboxylation of pyruvate to oxaloacetate.

It is possible to compensate partially the effect of low CO_2 tension for the growth of *Lactobacillus* with vitamin B_6 and with a sufficient pressure of CO_2 to suppress aspartic acid, necessary with low CO_2 pressures. Pyridoxal is perhaps one of the coenzymes of carboxylation which seems in some cases the last step of the amino synthesis, e.g. in the reaction phenylethylamine → phenylalanine.

Amination

The majority of microbes are able to utilize NH_3 as sole nitrogen source. This ability may be seriously diminished or lost as a consequence of mutations. It seems that the most common way by which NH_2 enters in the metabolism is the amination under the influence of specific aminases, e.g.

$$\text{(aspartase) fumarate} + NH_3 \rightarrow \text{aspartate.}$$

In the presence of specific (de)aminase (or (de)hydrogenase) α-ketoglutaric acid may be aminated, yielding an imino-acid which is hydrogenated to glutamic acid; the coenzyme is coenzyme II.

In the same way aspartic acid is formed from oxaloacetic acid and NH_3 under the influence of aspartic acid deaminase. It is important to note that an excess of aspartic acid decreases the need of biotin for lactobacillus and vice versa. On the other hand, biotin is important for yeast in the ammonia assimilation.

Glutamic and aspartic acids are able to be amidated to glutamine or asparagine, and act as reserves of $—NH_2$ groups. When *Clostridium cylindrosporum* oxidizes uric acid in the presence of labelled CO_2, the labelled carbon is found in the CH_3 and the COOH groups of acetic acid, and only in the COOH of glycine. Thus the synthesis of glycine is not, or not always, a mere amination of acetic acid. This is consistent with this hypothesis that CO_2 and NH_3 could react to give cyanic acid, the combination of formaldehyde and cyanic acid and subsequent reduction yielding glycine. Cyanic acid is perhaps important in amino-acid synthesis.

Transamination

Glutamic acid is the NH_2 donor for transaminating reactions:

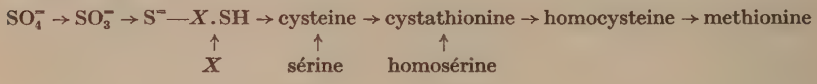
$$\text{glutamate} + \text{oxaloacetate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{aspartate},$$
$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{alanine}.$$

Specific apoenzymes and pyridoxamine- or pyridoxal-phosphate (or pyridoxal + ATP) are necessary.

Glutamic acid acts as $\text{—}NH_2$ donor, aminating pyridoxal into pyridoxamine which then transfers its amino group to a keto-acid. Vitamin B_6 has been found necessary for the metabolic conversion of anthranilic acid and indole into tryptophane (*Lactobacillus arabinosus*) and also for the synthesis of lysine and threonine and seem therefore to be a fundamental coenzyme in the synthesis of many amino-acids.

Synthesis of sulfhydryl group and of sulfhydrylated amino-acids

Evidence so far obtained indicates following steps in biosynthesis of cysteine and methionine:



$$\begin{tikzcd} SO_4^{=} \arrow[r] & SO_3^{=} \arrow[r] & S^{=}\text{—}X.SH \arrow[r] & \text{cysteine} \arrow[r] & \text{cystathionine} \arrow[r] & \text{homocysteine} \arrow[r] & \text{methionine} \\ & & X \arrow[u] & \text{sérine} \arrow[u] & \text{homosérine} \arrow[u] & & \end{tikzcd}$$

Assimilation of Amino-acids by Bacteria. By E. F. Gale

The proteins of bacteria resemble those of other tissues in being composed of some twenty-one amino-acids which must, consequently, either be synthesized or assimilated ready-made by the organisms concerned. Some bacteria are able to synthesize all their amino-acid requirements, while others have lost this ability and consequently are dependent upon the assimilation of some amino-acids from the external environment. Studies of the assimilatory processes have shown that Gram-positive bacteria not only assimilate certain amino-acids from the environment but also concentrate them in the free state in the internal environment prior to metabolism.

Considering first the assimilatory process: an amino-acid may pass across the cell-wall either by diffusion or as a result of an active process on the part of the cell. Lysine, a positive ion under physiological conditions, appears to cross the cell-wall by diffusion, and the amount which concentrates within the cell is determined by the negative charge within the cell. The passage of glutamic acid across the cell-wall requires energy which can be supplied by some exergonic metabolism such as glycolysis. The two processes can be clearly differentiated in the following ways:

(1) Lysine enters the cell in the absence of any other substrate; glutamic acid will only enter if a source of energy such as glucose is also supplied.

(2) If the rate of entry into the cell is studied at varying external concentrations, then lysine is found to enter at a rate which is proportional to the external concentration, while the rate at which glutamic acid enters the cell varies with the external concentration in a manner suggesting the variation of an enzyme reaction with substrate concentration.

(3) The temperature coefficient of the rate of lysine entry is the same as that for free diffusion of lysine; that for glutamic acid is markedly higher than for free diffusion.

The power to assimilate and concentrate free amino-acids appears to be restricted to Gram-positive species, although there may be wide differences in the degree of concentration achieved in the cells of different organisms. There are wide differences also in the assimilation affinities of different species. Of the organisms studied so far, *Staph. aureus* has the greatest affinity for, and capacity to concentrate, glutamic acid. The concentration within the internal environment is dependent upon the intact nature of the cell-wall, since lysis of the wall with tyrocidin or other detergent substances results in the release of the amino-acids, but not of proteins, from the internal environment.

The level of free amino-acid attained within the cell is determined by the balance between the rate at which the amino-acid is assimilated from the external environment and the rate at which it is metabolized within the cell. Consequently, anything which interferes with the assimilatory process but not with the internal metabolism will lead to a fall in the internal level, e.g. penicillin and glycolysis inhibitors act in this way. Conversely, after the addition of any substance which inhibits the internal metabolism without affecting assimilation, the internal level will rise to a saturation level. Studies of the internal metabolism have shown that glutamic acid concentrated within the cell acts as a source of that amino-acid for (*a*) condensation into protein in the growing cell, and (*b*) some other form of metabolism which is possibly transamination and which is inhibited by dyes of the triphenylmethane series. The rate of assimilation of glutamic acid from the external medium appears to be constant throughout the period of growth; the rate of condensation of the internal glutamic acid into protein is greatest during the early phases of growth and ceases when active cell division ceases; the rate of the dye-inhibited metabolism is greatest towards the end of the growth period, falling rapidly to zero after the cessation of growth.

The Gram-positive cell thus appears to have evolved a specialized cell-wall which, by allowing the internal concentration of amino-acids prior to their metabolism, compensates for the loss of synthetic abilities by the cell. Whether the Gram-negative cell accomplishes synthesis of amino-acids on the outside of the cell-wall and then assimilates the synthesized amino-acids, or whether it possesses completely different anabolic mechanisms is not known. It is, however, certain that the Gram-negative cell cannot effect an internal concentration of lysine or glutamic acid.

Peptides. By R. H. NIMMO SMITH

In 1941 Woolley (see Sprince & Woolley, 1945) found that group A haemolytic streptococci would not grow at all on a synthetic medium, and group D organisms only after a prolonged lag period, unless concentrates of a factor or factors were added. The factor was later named strepogenin. It is required for optimal growth by a number of organisms, but the effect is only seen with a small inoculum and in the early stages of incubation. It can be liberated from highly purified proteins by trypsin, and its properties suggest that it is a peptide containing glutamic acid. Serylglycyl glutamic acid has strepogenin activity for *L. casei*. Further evidence for a relationship between this tripeptide and strepogenin is provided in an interesting way. The tomato-wilting agent of the pathogenic fungus *Fusarium lycopersici* (lycomarasmin) is a peptide containing aspartic acid. Preparations of this toxin inhibit the growth of *L. casei*; both this inhibition and the toxicity in the tomato leaf are reversed by strepogenin. The toxic effects of natural lycomarasmin are imitated by serylglycyl aspartic acid and are reversed by both strepogenin and serylglycyl glutamic acid.

In spite of its wide distribution little is known of the metabolism of glutathione in micro-organisms. Gould (1944), however, has shown that strains of *N. gonorrhoeae* which have been subcultured daily for some weeks on a medium containing meat infusion develop a requirement for glutathione, although on first isolation they grew well in its absence. He also obtained evidence that such strains could be trained to dispense again with the factor. Isoglutathione and asparthione could replace glutathione to a certain extent. Cysteine, however, showed an inhibition which was reversed in a competitive manner by glutathione.

p-Aminobenzoic acid (*p*-AB) is involved in peptide linkage with glutamic acid in several interesting compounds. For one strain of *Sbm. plantarum* which requires *p*-AB for growth, Auhagen (1942) found that *p*-aminobenzoyl glutamic acid was considerably more active as a sulphanilamide antagonist than *p*-AB. This observation, however, has not been confirmed with other organisms; moreover, there is evidence that in the absence of sulphanilamide the relative activities of the two compounds are reversed.

p-Aminobenzoyl glutamic acid is also a moiety of the pteroylglutamic acid molecule (Angier *et al.* 1946). This compound can replace natural 'folic acid' in those organisms for which the latter is a growth factor (e.g. *L. casei*). An interesting possibility is that it is the incorporation of *p*-AB into the folic acid molecule that is blocked competitively by the sulphonamides; support is lent to this hypothesis by the work of Lampen & Jones (1946). These workers found that in a group of enterococci whose growth was stimulated by PG, sulphonamide inhibition was antagonized in a non-competitive way by PG, and that the organisms were highly resistant to sulphonamides in the presence of PG at a concentration no higher than that which gave growth stimulation.

'Conjugates' of folic acid with further glutamic acid residues have been isolated from natural sources. The 'fermentation *L. casei* factor', isolated from the fermentation residue of an unidentified diphtheroid, contains in all seven glutamic acid residues; enzymes exist in animal tissues and probably in some bacterial cells, which are capable of liberating free folic acid from this conjugate. Such enzymes have been termed 'conjugases' and, in view of the structure of the conjugate, are probably carboxypeptidases (Pfiffner *et al.* 1946).

Another peptide of *p*-AB, with ten or eleven glutamic acid residues and a third amino-acid which has not yet been identified, has been isolated from yeast by Ratner, Blanchard & Green (1946). It is natural to conjecture some relationship between this compound and the folic acid group, but so far no clue has been obtained to the biological role of this peptide, which accounts for 20–30 % of the total *p*-AB of yeast. It was not possible to show any growth-promoting or anti-sulphonamide activity, except in so far as the test organism may have been able to liberate *p*-AB by hydrolysis. It is possible, however, that such a large molecule may not be able to enter the bacterial cell. Sims & Totter (1947) have found that this peptide can inhibit, possibly competitively, the liberation of folic acid from its hexaglutamyl conjugate by the conjugases of rat liver and chicken pancreas.

The general properties of bacterial peptidases have been extensively studied (Johnson & Berger, 1942), and in several cases correspond closely to those of animal origin. On the other hand bacteria differ from other organisms in possessing peptidases whose optimum *p*H is in the acid range. Moreover, many bacteria contain peptidases capable of rapid hydrolysis of *d*-peptides. Doyle (1943) has concluded that the protozoon *Didinium* has lost the power of synthesizing a necessary peptidase, which it now derives from its living prey (*Paramecium*).

Several antibiotics are of peptide nature; a striking feature in this connexion is the relatively large proportion of *d*-amino-acids which they contain.

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Amino-acids in the Physiology of Bacteriophage. By JACQUES MONOD

During the past few years various types of effects have been reported, where certain amino-acids appear to play an important part either in phage multiplication within, or in phage adsorption on, a sensitive host.

(1) Viruses T4 and T6 were shown by T. H. Anderson, to require *l*-trypto-

phane as a 'co-factor' for adsorption on the host, *E. coli* B. *l*-Tryptophane activates the virus, not the host, and is required only for the adsorption, not for the multiplication of the phage. Recent observations by Delbrück appear to show that mutations of viruses from 'co-factor dependances' to 'co-factor independence' may occur.

(2) Another type of effect has been recently observed, where an amino-acid (*l*-leucine) although not required for the adsorption of the phage appears to be active in reducing the length of the 'latent phase' of phage multiplication, although the phage and the host will multiply in the absence of *l*-leucine or any other amino-acid.

(3) The third problem, which is the most complex as well as the most intriguing, has been raised by the discovery (E. H. Anderson, 1944; Elie Wollman, 1946) that *single* spontaneous mutations of bacteria may involve both a loss of sensitivity to certain phages, and a loss of the capacity to synthesize tryptophane, or proline, or sometimes both compounds. It was suggested by Anderson that these mutations affected an enzyme involved in the formation of a compound essential for the synthesis of tryptophane and for the synthesis of the specific 'receptor substance' responsible for phage adsorption.

Unfortunately, this simple and rather tempting scheme breaks down when confronted with too many facts, that is to say, when complex mutations involving several characters are studied. Luria suggests that a more likely mechanism may be one by which several mutations at different loci could occur together, by a deeper change in some material centre carrying the hereditary determinants. It is suggested that this deeper change might consist of a deficiency brought about in a chromosome-like structure.

Results practically uninterpretable with Anderson's scheme can be shown to fit rather well under that hypothesis.

For the time being each of these different 'amino-acid effects' constitutes a problem by itself. Trying to trace them all back to a common origin would be pure guess-work. But it is hoped that considering them side by side may lead to an interesting discussion.

Biochemical Genetics of Amino-acids. By G. Pontecorvo

ORIGINAL PAPERS

Microbial Transformation of Hydrocarbons. By Claude E. ZoBell

Bacteria and allied micro-organisms which synthesize, oxidize, or otherwise modify various kinds of hydrocarbons play an important role in the genesis or transformation of petroleum or its products.

Mixed cultures of marine anaerobes growing in mud tend to convert the remains of plants and animals into substances which are more and more hydrocarbon-like. This they do by liberating nitrogen, oxygen, sulphur, and phosphorus from organic compounds, thereby producing compounds containing proportionately more hydrogen and carbon. For example, the carbon content may be increased from an initial value of 52 % to as high as 75 % and the hydrogen content increased from an initial value of 6 % to as high as 10 % after eight month's incubation at 27° C., while the nitrogen, oxygen, sulphur, and phosphorus content decreased proportionately.

The synthesis of hydrocarbons ranging from $C_{16}H_{34}$ to $C_{30}H_{62}$ by pure cultures of *Desulfovibrio* has been demonstrated in a mineral salts solution enriched with *n*-capric acid as the sole source of energy. Lactic, caproic, palmitic, and stearic acids have also been used in such experiments. The yields of hydrocarbons have been very small (less than 0·1 % of the available carbon). The hydrocarbons were identified by chemical assay, ultra-violet absorption, infrared absorption spectra, and by the microscopic examination of crystals.

Preliminary surveys indicate that, with the exception of methane, hydrocarbons in minute amounts are produced by very few micro-organisms. Methane is known to be produced in abundance by several anaerobic bacteria. There are also unconfirmed reports for the bacterial synthesis of ethane, propane, benzene, and toluene. We have demonstrated the formation of phenol and *p*-cresol from tyrosine and/or phenylalanine but no benzene or toluene. Carotenoid and other unsaturated hydrocarbon pigments are produced in small amounts by certain bacteria.

Hydrocarbons are decomposed by a good many micro-organisms. More than a hundred species representing about thirty different genera have been shown to be able to attack one or more kinds of hydrocarbons. This has been demonstrated experimentally by various procedures, including (*a*) multiplication in mineral media containing HC's as the only source of energy, (*b*) oxygen consumption and CO_2 production in similar media, (*c*) disappearance or modification of HC's, and (*d*) the formation of acids or other metabolic products.

When HC's are dispersed in aqueous media by emulsification or on solid surfaces, virtually all types of gaseous, liquid, and solid HC's are susceptible to microbial modification. In general, paraffinic or aliphatic compounds are attacked more readily than homologous aromatic or naphthenic HC's. Unsaturated compounds are attacked more readily than saturated HC's. Provided the compounds are comparably dispersed, high-molecular weight paraffins are attacked more readily than short chains, and branched-chain compounds are more susceptible to attack than normal or straight-chain compounds. Thus *iso*-octane is oxidized more rapidly by bacteria than *n*-octane. Aromatic and naphthenic HC's having side chains appear to be attacked more readily than similar homologues without side chains.

Petroleum and its products, including illuminating gas, petroleum ether, petrol, kerosene, mineral oil, lubricating oil, petrolatum, paraffin waxes, asphalts, and tars are attacked by micro-organisms under favourable conditions. Even 'antiseptic' HC's such as benzene, toluene, xylene, naphthalene, anthracene, and related compounds may be destroyed by micro-organisms in soil or marine mud. The bacterial destruction of carcinogenic HC's may prove to be of special interest. The microbial destruction of the HC's in both natural and several kinds of synthetic rubber has been demonstrated.

The principal products resulting from the microbial assimilation of HC's are CO_2, water, cell substance, organic acids, esters of fatty acids, alcohols, and pigments. Only the first three have been produced in abundance.

Besides producing and assimilating HC's, bacteria may contribute to the liberation or separation of petroleum from oil-bearing sediments. Laboratory investigations indicate that they may do this (*a*) by dissolving calcareous and sulphate rocks, (*b*) by producing CO_2 which decreases the viscosity of oil, (*c*) by displacing films of oil physically, (*d*) by producing gases which force oil out of micro-traps, and (*e*) by producing detergents or surface-active substances. In one or more of these ways bacteria may have contributed to the migration and accumulation of crude oil in past geological ages and they may find important applications to increase the secondary recovery of oil from oil-bearing formations in the future.

p-Aminobenzoic Acid and Folic Acid Derivatives in Relation to Bacterial Growth and Sulphonamide Action. By R. H. Nimmo Smith and D. D. Woods

p-Aminobenzoic acid (*p*-AB) is an essential growth factor for many bacteria. If its only function is for the synthesis of folic acid (which contains a *p*-AB residue), it is possible that micro-organisms requiring *p*-AB for growth would grow if provided directly with folic acid. Pteroylglutamic acid (a synthetic member of the folic acid group) was found to replace *p*-AB for the growth of a selection of organisms requiring the latter (*Clostridium acetobutylicum*, *Acetobacter syboxydans* 621, *Streptobacterium plantarum* spp., and an induced mutant of *Neurospora crassa*). In all cases, however, the molar concentration of pteroylglutamic acid required was 10–100 times that of *p*-AB. With a mutant *Bact. coli*, Lampen, Roepke & Jones (1946) found this substance essentially inactive in replacing *p*-AB. Synthetic pteroic acid also replaced *p*-AB with the test organisms but again larger quantities (2–10-fold) were required.

Pteroylglutamic acid behaved also as an anti-sulphonamide agent with all the above organisms. With *Cl. acetobutylicum* and the strains of *Streptobacterium plantarum* the antagonism was non-competitive, the concentration sufficing to support growth doing so irrespective of the sulphonamide concentration. It is therefore likely that the main action of the drug on these organisms is to inhibit the synthesis of folic acid from *p*-AB. A similar conclusion was reached by Lampen & Jones (1946) with regard to several species of enterococci with which folic acid, though not essential, stimulated growth.

With *Acetobacter suboxydans* and the *Neurospora* mutant, pteroylglutamic acid antagonized sulphonamides in a competitive manner. With these organisms it appears that utilization for the synthesis of folic acid may not be the only function of *p*-AB, or that synthetic pteroylglutamic acid is not identical with (or convertible to) the natural folic acid of these organisms. There has been evidence recently (e.g. Hall, 1947) that pteroylglutamic acid may not be identical with all natural non-conjugated members of this group.

Pteroic acid gave competitive antagonism of sulphonamide inhibition of all the test organisms except *Cl. acetobutylicum*. With the latter, antagonism was non-competitive at concentrations of 10^{-4} M-sulphanilamide (or 10^{-6} M-sulphathiazole) and above, but competitive at lower concentrations.

Under conditions in which multiplication did not occur, and in the presence of *p*-AB, *Streptobacterium plantarum* synthesized folic acid as estimated by the growth response of *L. casei* and *Strept. faecalis* R. The synthesis is inhibited by sulphanilamide. This technique is being used for more detailed studies of the conversion of *p*-AB to folic acid.

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On Bacteria-like, Gliding, Filamentous Organisms. By E. G. Pringsheim

The order Bacteria contains, apart from the true Bacteria, a number of groups, the relationship of which is a matter of controversy, for instance the Actinomycetes, the Mykobacteria, the Chlamydobacteriaceae, the Beggiatoaceae, the Myxobacteria. Of these the Myxobacteria, and still more the Beggiatoaceae are to my mind closer to the Cyanophyceae than to the Eubacteria, owing to their gliding movement.

In fact *Beggiatoa* is not the only colourless, gliding, filamentous organism, but the others are not so conspicuous because of their lesser size and by never being amassed in great numbers. They are found as single threads or as small groups and bundles between other organisms. However, these, often very small microbes are by no means rare. They have been found by me in Austria, in Bohemia, in Cambridgeshire and in Westmorland, that is everywhere they have been looked for. Bottom deposits in quiet waters with a moderate amount of decaying organic matter generally contain them. They are associated with Cyanophyceae, iron bacteria, *Beggiatoa* and with various flagellates. Some were found in cow dung. Those species, which were isolated in pure cultures, *Beggiatoa* and others, multiply on agar with protein derivatives and less well in solutions. The growth on agar consists of more or less spirally arranged filaments and curls. These organisms do not seem to have characteristic biochemical properties.

Morphologically the filaments differ in width and length of the cells, and in the kind of movement. Some of them are similar to the thinner forms of *Beggiatoa* where these occur in nature without sulphur droplets, apparently living on organic compounds, and may in fact be related to them. The narrowest species are less than 1 μ across, the thickest, apart from *Beggiatoa*, up to $2\frac{1}{2}\,\mu$

wide. The ratio length to width of the cells seems to have some relation to the kind of movement and the bending of the threads. The tendencies to form long filaments or only short hormogonia-like cell rows are also very different.

It will take some time to bring some order into this new group of little known creatures. Even in *Beggiatoa*, which has been the object of much physiological curiosity, the differentiation of the genus into species is quite arbitrary, specific names having been given to size-groups characterized by a lower and an upper boundary in width.

These organisms are definitely not Bacteria although they would be mistaken for such in dried and stained preparations. Their relationship to the Cyanophyceae seems to be nearer than that to the Myxobacteria but I am inclined to consider at least part of them as belonging to a separate natural group. The reason is that the kind of locomotion in some of them is different from anything known in the Cyanophyceae, either by its celerity or by hingelike bending at the cell boundaries.

Biochemical changes underlying the Assay of Nicotinic Acid, by Titration with Alkali of Cultures of *Lactobacillus arabinosus*. By Henry McIlwain and Douglas A. Stanley

The method of assay chosen for investigation was based on those of Snell & Wright (1941), Krehl, Strong & Elvehjem (1943) and Barton-Wright (1946). In the assay, *L. arabinosus* 17/5 was grown for 3 days in several portions of media, some of which contained nicotinic acid (from 15 to 300 $\mu\mu$mol./ml.) and others contained materials being assayed. After growth, the quantities required of standard NaOH to restore the cultures to their original pH were determined. The relation between the quantities of nicotinic acid added and NaOH required in the several tubes, was to a large extent a linear one, with positive intercept on the NaOH-axis and, beyond *c.* 200 $\mu\mu$mol./ml., concave towards the nicotinic acid-axis. NaOH-titres increased most rapidly during the first 2 days and after 3 days changed relatively little.

We have investigated the relationship between nicotinic acid and NaOH-titre and give below our interpretation of the following of its characters: (1) the intercept; (2) the linear portion, and its slope; (3) the stability of the readings which give this slope, after a finite time; (4) deviations from linearity; (5) the greater regularity found in NaOH-titre than in mass of growth, a regularity favouring the assay method described rather than a turbidimetric method.

Character (1). Circumstantial evidence supported the general opinion that this was due to the presence of nicotinic acid or its derivatives, in the basal medium or inoculum.

Character (2) appeared due to a bacterial system in which a reaction of some 20 μmol./mg./hr. in glucose was associated with one of some 0·1 mμmol./mg./hr. in nicotinic acid or a derivative of it (cf. McIlwain, 1946*b*, 1947), the ratio between their rates being fairly constant though the absolute values of both could vary considerably. Character (2) was not due to a given quantity of nicotinic acid producing only a given quantity of organisms which then died or became generally enfeebled. The quantity of organisms produced from a given quantity of nicotinic acid has been varied experimentally while NaOH-titres remained constant.

Character (3). Although this ratio was maintained, the rates of both μmol. and mμmol. processes fell during assay and were very much lower on the 4th than on the 1st day. This was due to a specific deficiency induced in the organisms during the assay by the mμmol. change. It could be made good by nicotinic acid, independently of growth.

Character (4). The deviation at higher nicotinic acid concentrations appeared due to much of the bacterial activity (in the sense of McIlwain, 1946*a*) being brought about by cells during a time when the μmol. reaction was not being limited by the mμmol. change.

Character (5) follows from observations under (2) and (3).

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Some Biological Properties of Penicillin II(G) and Penicillin IV(K)

By J. Ungar

Apart from differences in the chemical structure of the two penicillins, they vary also in their biological activities. Micro-organisms seem to be less susceptible to K than to G, with the noticeable exception of strains of *Staphylococcus*. Still more marked are the variations in behaviour of the two types of penicillin when injected into different animals (rabbits, guinea-pigs and mice). We have confirmed that the curative effect of penicillin K for mice infected with *Streptococcus haemolyticus* or *Spirochaeta recurrentis* is about 25–30 % of that of penicillin G. Rabbits injected intramuscularly with 20,000 units of penicillin show a lower penicillin level in the blood, which is also of shorter duration, after K than G. Guinea-pigs injected intramuscularly with 4000 units of penicillin G show penicillin in the blood for 2 hr., while with K no penicillin is detectable after 1 hr. Penicillin K disappears even more quickly from the blood of mice. Mice injected with 150 units of penicillin G show blood levels up to 2 hr., whereas after injection of penicillin K, hardly any penicillin is detectable after ½ hr.

Penicillin K is excreted in the urine of the animals mainly during the first 2 hr. and the total excreted in 24 hr. is about 20–25 % of the injected amount. Penicillin G is known to be excreted in the urine over a period of 7–10 hr. and the total excreted in 24 hr. is 50–75 %.

We investigated the effect of blood serum from rabbits, guinea-pigs and mice. The effects of extracts of macerated liver, kidney, lung and spleen (ground to a fine pulp with acid-washed silver sand in a porcelain mortar and extracted with water at 4° C. for 2 hr.) were also studied. Guinea-pig and mouse serum inactivated penicillin K at a higher rate than penicillin G; the action of rabbit serum was less marked. Liver (from all three species) gave extracts destructive of penicillin K, but less so of penicillin G; extracts of other organs were much less active. The guinea-pig liver-extract invariably completely inactivated penicillin K in about 15 hr., while in the same period penicillin G was only reduced to about half its original activity.

	Penicillin G			Penicillin K		
Extract	0 hr.	7 hr.	15 hr.	0 hr.	7 hr.	15 hr.
	Units present in 1 ml. of extract					
Mouse liver I	50	50	45	50	50	20
Mouse liver II	50	50	45	50	50	25
Mouse serum	50	50	53	50	20	8
Guinea-pig liver I	50	45	25	50	7·5	Nil
Guinea-pig liver II	50	45	25	50	10	Nil
Guinea-pig serum	50	45	25	50	45	20
Rabbit liver I	50	50	30	50	50	12·5
Rabbit liver II	50	50	40	50	50	20
Rabit serum	50	45	20	50	20	8
Saline control	50	50	50	50	50	45

I and II refer to averages of two experiments.

The penicillin assay was carried out by the dilution method using *Staph. aureus* as a test organism.

B. subtilis penicillinase completely inactivates both penicillin G and K. The factor in the liver responsible for the inactivation of penicillin is heat labile (55° C. for 30 min.), is adsorbed on charcoal and is non-dialysable.

The evaluation of penicillin blood levels in mice can be used as a crude method of differentiating batches of commercial penicillin with a high or low content of penicillin K, as seen in the table below:

	Blood levels					
Mice injected with	15 min.	30 min.	1 hr.	1½ hr.	2 hr.	3 hr.
250 units of K	0·125	0·06	—	—	—	—
250 units of K and G in equal parts	2	2	0·25	0·03	—	—
250 units of G	4	4	1·5	0·25	0·1	—

So long as mixed penicillins are used therapeutically it is obviously advantageous to have a test to complement existing laboratory methods for evaluation of the therapeutic activity of commercial penicillin. The estimation of the duration and titre of blood levels in small animals (particularly mice), the effect of liver extract on penicillin, and the establishment of the curative effect in infected mice, can be used for this purpose.

The penicillins G and K were purified by our colleague, Dr E. Lester Smith, by the countercurrent partition method and by crystallization of the organic base and sodium salts. They assayed 98 % G and 99 % K respectively by paper strip chromatography (Goodall & Levi, 1946).

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Factors affecting the Synthesis of Glutamic Acid by Yeast Cells.

By E. S. Taylor

Investigations on the amino-acid composition of yeasts have shown that these cells contain relatively large amounts of certain amino-acids in the free state in the internal environment (Freeland & Gale, 1947; Taylor, 1947). The concentration attained is to a certain extent dependent on the concentration of amino-acids present in the growth medium, but free amino-acids are found in the internal environment when the cells are grown in a medium devoid of amino-acids; consequently the appearance of free amino-acids within the internal environment can be used as a means of studying amino-acid synthesis.

The yeast used for this work grows rapidly and in heavy crop in a medium containing amino-acids and effects a very high concentration of glutamic acid inside the cells during such growth. If, however, the cells are incubated with glucose in a suitable salt mixture for 3–4 hr. after harvesting, this internal glutamic acid is metabolized and the internal concentration falls to a low level. This method of producing cells with a low internal concentration of free glutamic acid is experimentally more convenient than the much slower method of growth in an amino-acid-free medium.

If such depleted cells are incubated in salt mixture containing glucose and ammonia, then glutamic acid is synthesized and concentrates within the cells; omission of either glucose or ammonia stops the synthesis. If the cells are subjected to increased washing during the preliminary incubation with glucose then the synthesis becomes erratic and may cease altogether, but can be restored by addition of the bios complex to the reaction mixture. Analysis of the factors in bios needed for the synthesis shows that after a moderate degree of washing, panthothenic acid alone can replace the bios mixture, the other factors having no stimulatory effect. The effect of the bios mixture can be largely antagonized by pantoyl taurine, and at this stage ammonia assimilation and fermentation are normal. On more thorough washing it is found necessary to add biotin to the glucose, ammonia and pantothenic acid mixture before glutamic acid synthesis will occur. The cells at this stage are unable to assimilate ammonia normally, and the action of biotin is to restore this ability as previously shown (Winzler, Burk & du Vigneaud, 1944). Exhaustive washing of the cells leads to an impairment of the fermentation system which can only be made good by addition of the remainder of the bios complex.

It appears that continued washing of the cells results in the progressive removal of growth factors, enabling identification of the following steps essential to the intracellular synthesis of glutamic acid:

(1) Glucose fermentation as the source of carbon and energy; requires the presence of glucose, nicotinic acid, aneurin and, possibly, pyridoxin and inositol.

(2) Assimilation of ammonia as the source of nitrogen; involves glucose fermentation and the presence of biotin.

(3) Synthesis of free glutamic acid within the cell; requires pantothenic acid.

When the complete system is present, free glutamic acid is synthesized and concentrates within the cell. Analysis of free and combined glutamic acid in the cell shows that the early stages of synthesis are not accompanied by condensation of the glutamic acid into peptides, but that this begins after about 2 hr. under the experimental conditions used.

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DEMONSTRATIONS

E. G. Pringsheim. ‘Bacteria-like, gliding, filamentous organisms.’

R. Barer. ‘Optical micro-dissecting apparatus, including a reflecting microscope, phase contrast microscopy, and 35 mm. micro-cinematography.’

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Sixth General Meeting (which was also the Annual General Meeting) in the Bonnington Hotel, Southampton Row, London, W.C. 1. The meeting consisted of (a) a Symposium on 'Microbial Association: Soil, Water and Sewage' on Tuesday, 23 March 1948, *and (b) a reading of original papers on Wednesday, 24 March* 1948. *The following communications were given:*

COMMUNICATIONS

MICROBIAL ASSOCIATION: SOIL, WATER AND SEWAGE

Microbial association in soil. By H. G. Thornton

Production of antibiotics in relation to the ecology of soil-inhabiting micro-organisms. By P. W. Brian

1. The commoner species among the Protozoa, bacteria, actinomycetes and fungi of the soil have a wide distribution and constancy of occurrence. It is possible, therefore, to speak of a characteristic soil association of micro-organisms. A considerable proportion of these organisms possess the power of producing antibiotic substances in artificial culture.

2. In many well-established cases of antagonism between saprophytic soil organisms and soil-borne plant-pathogenic fungi, considered as examples of one type of microbiological balance in soil, one finds that all the observed facts can be explained by assuming the production of antibiotic substances by the antagonist, which have a suppressive action on the multiplication or metabolic activity of the pathogen. In certain cases there is strong evidence in favour of this explanation.

3. Evidence that organisms capable of producing antibiotics in pure culture can do so in soil is not strong. Nevertheless, there are indications that they are so produced under certain conditions. Consideration of the stability in soil of certain pure antibiotics indicates that instability cannot be regarded as being a factor precluding them from biological significance in soil.

4. Those antibiotic substances produced by organisms common in soil and which are relatively stable chemically are most likely to be of significance in connexion with biological equilibria in soil. On this basis those known antibiotics most worthy of study can be indicated.

Soil Myxobacteria. By B. N. Singh

Myxobacteria were recognized as an independent group by Thaxter in 1892. Previously they were considered to be dung organisms and rare in soil.

The studies on the selection of bacterial food by certain groups of soil micro-predators led to the development of a method of isolating, purifying and roughly estimating the numbers of myxobacteria from soil and other substrates. *Myxococcus virescens*, *M. fulvus*, *Chondrococcus exiguus* and *Archangium* sp. are widely distributed in the soils of Great Britain. *Myxococcus virescens* and *Chondrococcus exiguus* seem to be common in soil samples obtained from Sweden, Holland, Jan Mayen, Canada and South Africa. The common occurrence of myxobacteria in Rothamsted soils which have been unmanured or treated with artificial fertilizers only for over 100 years proves that they are true soil inhabitants. They have been found in numbers ranging from 2000 to 76,000/g. soil.

Ninety-four varied strains of bacteria were given as food to three species of myxobacteria (*Myxococcus virescens*, *M. fulvus* and *Chondrococcus exiguus*) on non-nutrient agar. Under these conditions, a higher percentage of Gram-negative than Gram-positive bacteria are attacked. Pigmented bacteria are more often resistant than non-pigmented. It has been conclusively proved that, at least on solid media, certain myxobacteria lyse living Gram-negative bacteria and do not merely attack dead bacterial cells. A method of growing myxobacteria in mass liquid culture to produce extracellular lytic secretions has been developed.

Dr A. E. Oxford has found that a strain of *Myxococcus virescens* isolated at Rothamsted produces two substances: one is a soluble non-enzymatic antibiotic substance, and the other is an exocellular lytic (proteolytic) enzyme which is active against non-viable bacteria only. Thus myxobacteria provide excellent material in which the mechanism of antibiotic action may be analysed.

Micro-organisms and soil structure. By R. J. Swaby

The physical condition of soils is improved by addition of readily decomposable organic materials. Microbial cells and metabolic products restore soil structure by binding the soil particles into friable crumbs or aggregates capable of resisting the action of water.

In pure culture studies it was found that the aggregating power of micro-organisms was as follows: fungi > actinomycetes > capsulated and gum-producing bacteria > yeasts > non-capsulated bacteria. Fungal hyphae entangled the soil particles into stable aggregates. Weaker crumbs were formed by the frail threads of actinomycetes. A few bacteria produced gums capable of gluing soil particles into water-stable aggregates, but the majority of bacterial slimes were almost useless because they remained water-soluble after drying. Most bacteria had no effect on soil aggregation.

Mixed cultures of micro-organisms were used to elucidate effects obtained in the fields. Mixed inocula of fungi or actinomycetes gave slightly better aggregation than pure cultures, but neither capsulated nor non-capsulated bacteria in mixtures gave better results than single strains. More complex mixtures containing fungi, actinomycetes and bacteria gave good aggregation sometimes, but poor results at others. If the mixture contained antagonistic bacteria which inhibited the growth of either the fungi or actinomycetes, then few crumbs were formed. Effects obtained in the field were finally imitated in the laboratory by inoculating sterilized soil with complex mixtures of fungi, actinomycetes and bacteria.

Aggregates stabilized by mycelia do not last very long in the field because the hyphae are decomposed by bacteria. The bacterial metabolic products derived from fungal threads do not cement the soil nearly as well as the hyphae.

(Lantern slides have been prepared illustrating all these points.)

The production of an antibiotic substance in the soil which inhibits phytopathogenic organisms. By E. GROSSBARD

An attempt was made to induce the production of antibiotic substances in the soil as a means of controlling soil-borne diseases.

Penicillium patulum is known to produce an antibiotic active against certain phytopathogens. When this fungus was cultured on sterilized soil the aqueous extract did not inhibit the test organisms, but when *P. patulum* or other antagonists such as *Aspergillus clavatus*, *A. terreus* and *Streptomyces antibioticus* were grown on autoclaved straw an antibiotic was readily formed. A mixture of sterilized soil, straw and water was inoculated with *Penicillium patulum*. The liquid expressed from the soil after incubation completely inhibited the growth of the phytopathogenic and other test organisms. The addition of glucose increased this activity. When straw was omitted and glucose mixed with the soil, activity was only slightly reduced. Thus carbohydrate decomposition may be one essential factor in the production of an antibiotic in sterilized soil by *P. patulum*.

The addition of carbohydrates to the soil is reported to reduce the virulence of certain diseases by modifying the microflora. It is possible that, in addition, this treatment may also induce antibiotic production in the soil not only by *P. patulum* but also by other micro-organisms.

The possibilities of a practical application will be outlined.

The ciliate protozoan fauna of soil and inland waters. By ERNEST GRAY

One year's study of the bacteria in the current core of a chalk stream has shown that there was a natural water bacterial flora (*Pseudomonas* sp., *Flavobacteriae*, *Micrococci* sp.) which was supplanted by a bacterial flora of soil types (*B. mycoides*, *B. subtilis*, *Actinomyces* sp., *B. coli* irregulars) after heavy rain, floods, or prolonged periods of hot dry weather, when presumably the banks continually crumbled into the stream.

It was also observed that bacteria-eating ciliates in the current core were most numerous and most varied when a soil bacterial flora predominated. Further, many of these bacteria-eating ciliates (e.g. *Colpoda*, *Halteria*, *Holophyra*) appeared in numbers after heavy rain, floods, and periods of hot dry weather, that is, after the same physical conditions which had ushered a soil bacterial flora into the stream.

Examination of the soil on the banks of the stream, and of the soil 200 yards away on the banks of gullies emptying into the stream showed the presence of ciliates many of which were indistinguishable from forms found in the stream (*Colpoda, Holophyra, Oxytricha, Halteria*). Ciliate Protozoa indistinguishable from forms found in the stream were also obtained from within dried mud originally excavated from the bottom of the stream and cast on adjoining land (*Oxytricha, Hypotrichid* sp., *Halteria*).

Soil ciliate Protozoa in artificial culture developed slowly—in 1–2 weeks—if the only food supply was the natural soil bacterial flora. If, however, the culture was inoculated with micrococci isolated from the stream, a massive development occurred within 3–5 days.

It is suggested that there is a free exchange between soil ciliate Protozoa and those of inland waters, and that any distinction drawn between them is apparent and not real; in short, water ciliates are identical with those of the soil, one habitat being the source of the other.

Microbial association in sewage. By L. A. ALLEN

Domestic sewage is a dilute fluid containing fats, carbohydrates, and proteins, together with products of decomposition of these materials, and a variety of inorganic salts. Some of these constituents are in solution; others are colloidally dispersed, and they may be augmented by those of a number of trade wastes. There is also a heterogeneous population of micro-organisms derived from the intestine and from soil and water.

Sewage is a nutrient medium and the conditions to which it is subjected determine the groups of micro-organisms which predominate, thus giving rise to different microbial associations. Some of these associations cause difficulties and economic losses, others are beneficial and form the basis of modern methods of purifying sewage and trade wastes.

Undesirable associations may arise, for example, in a long length of sewer, where sewage receives little or no aeration. Growth of the mixed flora reduces the oxidation-reduction potential to the point where sulphate-reducing bacteria are encouraged. The resulting trouble is not confined to an objectionable odour at the treatment works, for some of the hydrogen sulphide which escapes into the atmosphere above the liquid is absorbed by the moist surface of the sewer and, in the partially aerobic conditions which obtain there, it may be converted by sulphur-oxidizing bacteria to sulphuric acid, which attacks the concrete.

When sewage is subjected to aeration and agitation there develops an active biological material, which increases in amount if the sewage is continually or periodically replaced. Such material is found in the form of a slimy growth on the bottom and sides of a polluted stream, as a biological film on the surface of the medium in a percolating filter, and as gelatinous flocs in an activated sludge tank. These films or sludges consist of a mass of bacteria and fungal hyphae embedded in mucoid material, which also entrains a variety of Protozoa, and they are able to effect with remarkable rapidity profound changes in the composition of sewage with which they are brought into contact. The first stage—the clarification stage—of the reaction between the two results in adsorption of dissolved organic matter and flocculation and removal of the particles in suspension. In this way a highly clarified liquid may be produced in a matter of 20 or 30 min. from grossly polluted water flowing over slime-covered stones or from sewage percolating through a filter.

The second stage of the reaction consists of oxidation of organic matter by the micro-organisms in the sludge accompanied by synthesis of new cell material. This microbiological oxidation has been investigated by the Thunberg tube technique, by the microrespirometer, and by measuring absorption of oxygen, evolution of carbon dioxide, and reduction in biochemical oxygen demand during aeration of activated sludge with different substrates in a closed aeration system. Although bacterial cells are mainly responsible for the oxidation, bacteria-eating Protozoa appear to be necessary to maintain the activity of this microbial association at an optimum.

An example of a different kind of bacterial association, of which advantage was taken to avoid the discharge of a highly polluting trade waste, is that between aerobic and anaerobic bacteria in the aerated retting of flax. Species of *Clostridium* growing in the flax stems perform the actual retting; aerobic and facultative bacteria oxidize so effectively the organic matter in the liquid in which the flax is immersed that it can be reused for successive batches of flax.

The protozoan fauna of sewage. By A. Neville Barker

Protozoan associations of sewage disposal works appear to be determined by the complex influence of a number of environmental factors. Not only are there changes in these associations correlating with the degree of purification of the sewage, but superimposed on these are changes of a seasonal nature and changes due to local characteristics, e.g. type of aeration system employed and sewage strength.

Transitions throughout the system correlate with the nature of the food supply and the availability of oxygen. Thus, saprophytic Protozoa present in the early stages of purification are rapidly superseded by holozoic forms and, where a very pure effluent is attained, the presence of holophytic forms may be noted. Within the holozoic group transitions occur which appear to be related to the state of aeration or purity of the sewage and range from a pollutional association in which Amoebina and Mastigophora predominate to one characteristic of a high degree of purity in which Peritricha and *Paramoecium* predominate. Where the crude sewage is 'weak' these transitions may occur rapidly and an association characteristic of a high degree of purity may colonize the bacteria beds. On the other hand, when a strong sewage is treated the pollutional association may never be completely replaced.

Sewage strength also influences the nature of the seasonal changes in the bacteria beds so that a sewage of 'medium strength' may show a protozoan fauna which is limited by temperature in winter and by the predatory activities of a large insect population in summer. 'Weak' and 'strong' sewages may not support huge insect populations, so that temperature may have a direct effect on the fauna through most of the year or may act indirectly through the sloughing or off-loading of the beds. During sloughing the opening of the beds usually leads to increased purification and a consequent change in the protozoan association to one characteristic of a higher degree of purity, e.g. with a 'weak' sewage there may be restriction of variety and abundance of Protozoa and with a 'strong' sewage a change from the pollutional association with flagellates predominating to one in which ciliates increase in both variety and abundance.

In activated sludge systems the protozoan associations are constant throughout the length of the channels and are determined by the strength of sewage treated and the degree of purification attained.

The growth of fungi in percolating filters in relation to the strength of sewage treated. By T. G. TOMLINSON

In the standard process of biological filtration, one of the most important factors limiting the rate of treatment of sewage is the accumulation of fungus mycelium during the winter. The fungus is disintegrated by the renewed activity of the filter metazoa and in particular insect larvae in the spring and early summer which causes the discharge of large amounts of solid matter in the effluent over a short period.

By diluting sewage with an equal volume of filter effluent it was found that the rate of growth of fungal film was reduced to one-fifth and that growth ceased when the biochemical oxygen demand of the sewage was reduced to a value below 4 parts/100,000. Treatment with filter effluent causes a change in the internal organization of the fungal hyphae, which is followed by bacterial breakdown of the cell wall. Experiments with pure cultures of mycelium-decomposing bacteria showed that their activity is inhibited by undiluted sewage or by 0·2 % glucose but not by 0·02 % glucose, 0·1 % peptone, or by sewage diluted with three volumes of tap water.

Greatly increased rates of treatment of sewage are possible by the alternate treatment of a filter with sewage and partially purified filter effluent. The effect of this method is to reduce the rate of growth of fungi and to allow bacterial decomposition of the fungus mycelium to take place.

Micrococcin, an antibacterial substance from a micrococcus isolated from sewage. By T. L. SU

A strain of *Micrococcus* isolated from the sewage in Oxford was found to produce a substance active against many strains of Gram-positive bacteria including mycobacteria. The active substance extracted, named micrococcin, is soluble in alcohol, acetone and chloroform, insoluble in ether, and only very slightly soluble in water.

It inhibited at a dilution ranging from 1 in 640,000 to 1 in 64,000,000 the growth of *Streptococcus pyogenes*, *Staphylococcus aureus*, diphtheria bacilli, and Gram-positive spore-forming rods. It inhibited less powerfully the growth of

Streptococcus viridans, *Pneumococcus*, *Cl. welchii*, and *Myco. tuberculosis*, but nearly all Gram-negative organisms including *Neisseria* were insensitive to it.

Naturally resistant strains of susceptible species of Gram-positive cocci and also of tubercle bacilli were isolated.

No significant reduction of activity was observed in the presence of 50 % horse blood or serum. Alteration in the size of the inoculum made little difference to the concentration required to inhibit growth.

The synthesis of bacterial viruses in infected bacteria.

By Seymour S. Cohen

The synthesis of nucleic acid and protein was studied in *Escherichia coli* strain B, infected by T $2r^+$ or T $4r^+$ bacteriophage. Adsorption of virus stopped bacterial multiplication and enzyme synthesis without affecting respiration in a lactate medium. The metabolism of the infected cell was directed exclusively to the synthesis of virus substance, i.e. desoxyribosenucleic acid and protein. As determined by studies with radioactive P and other techniques, virus substance was synthesized for the most part after infection from compounds in the medium. Ribose nucleic acid was completely inert in the infected cell, being neither synthesized nor metabolized.

Protein synthesis began at infection; nucleic acid synthesis started 7–10 min. after infection. The amount of nucleic acid synthesized closely corresponded to the amount contained in the number of T 2 particles liberated at lysis. The rates of nucleic acid and protein synthesis were constant, suggesting that the synthesis of virus components was a function of the bacterial enzymes, being independent of the number of virus particles formed.

A case of bacterial symbiosis based on the combined growth-stimulating and growth-inhibitory properties of long-chain unsaturated fatty acids.

By M. R. Pollock

A chance bacterial contaminant, 'Q' (a so far unidentified diphtheroid), was found to have a remarkable growth-promoting effect on *H. pertussis*, enabling the latter to grow profusely in ordinary broth or on agar. The strain of *H. pertussis* used is unable to grow on tryptic meat agar unless supplemented by blood, albumen or charcoal, the main effect of which has been shown to be due to their power of absorbing toxic unsaturated fatty acids from the culture (1947). For 'Q', however, unsaturated fatty acids (oleic, linoleic or linolenic) are necessary growth factors, and even on ordinary tryptic meat agar the organism will grow only feebly unless oleic acid is added, while on 10 % blood agar the growth is even poorer and on charcoal agar undetectable. Mixed inocula of 'Q' and *H. pertussis* (in suitable proportions) will give rise to satellitism of *H. pertussis* colonies around those of 'Q' on agar and to satellitism of Q' around those of *H. pertussis* on charcoal agar. In each case the 'parent' colonies, though often apparently homogeneous, are, in fact, mixed colonies of both organisms. The most probable explanation is that oleic acid or some similar compound present in the medium and produced to some extent by *H. pertussis* itself is absorbed by 'Q'. One organism is thus protected from the harmful effect of a substance which is necessary for the growth of the other, and both benefit.

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ORIGINAL PAPERS

Lecithinase production by aerobic sporing bacilli. By H. P. Chu and C. A. McGaughey

Biochemical and immunological properties of lipolytic enzymes in clostridial toxins. By M. G. Macfarlane

Culture filtrates of various strains of *Cl. welchii*, *Cl. oedematiens* and *Cl. haemolyticum* contain *lecithinases* which have a similar biochemical action in decomposing lecithin with formation of phosphorylcholine. The lecithinases of *Cl. oedematiens* Type A and Type B filtrates are immunologically distinct from each other and from *Cl. welchii* lecithinase, in that their activity is inhibited by a homologous but not by a heterologous antitoxic serum; the Type A and Type B lecithinases are probably identical with *Cl. oedematiens* γ- and β-toxins respectively (Oakley, Warrack & Clarke, 1947; Macfarlane, 1942).

Cl. haemolyticum lecithinase is inhibited by *Cl. oedematiens* Type B antitoxin but not by Type A or by *Cl. welchii* antitoxin. The lecithinase activity of a crude *Cl. haemolyticum* toxin was high, comparable to that of toxigenic strains of *Cl. welchii* Type A; this supports the suggestion of Jasmin (1947) that the lethal capacity of *Cl. haemolyticum* toxin, which runs parallel with the Nagler reaction and the haemolytic capacity, is due, like that of *Cl. welchii* Type A toxin (Macfarlane & Knight, 1941), to the action of a lecithinase.

Cl. oedematiens Type A filtrates contain, in addition to the lecithinase, a *lipase* which is possibly responsible for the 'pearly layer' effect in colonies of this organism.

Culture filtrates of some strains of *Cl. sordellii* contain a lecithinase which is not fully characterized, together with another factor producing opalescence in egg-yolk emulsions.

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Synthesis of '*L. casei* factor' by *Streptobacterium plantarum*: competitive inhibition by sulphonamides. By R. H. Nimmo-Smith, June Lascelles and D. D. Woods

In his original hypothesis Woods (1940) suggested that the sulphonamides inhibit competitively the further utilization of *p*-aminobenzoic acid (*p*-AB). The publication of the structure of liver '*L. casei* factor' (Angier *et al.* 1946) and the experiments of Lampen & Jones (1946) suggest that it is synthesis of factors of the 'folic acid' group which is inhibited by the sulphonamides.

In the present experiments we have studied the synthesis of such a factor by resting suspensions of *Streptobacterium plantarum* 5S.

Some of the factor present in cells freshly harvested from a semi-synthetic medium disappears when they are incubated in the growth medium, lacking in *p*-AB. If these cells are now incubated in the presence of *p*-AB, a considerable increase (10–50-fold) in the factor can be detected.

Synthesis of the factor is just as good by cells suspended in buffer solution containing glucose, *p*-AB and DL-glutamic acid; moreover, under these conditions no growth occurs.

In the absence of glucose only a small amount of the factor is synthesized; the higher the initial glucose concentration (up to 0·2M), the longer does synthesis of the factor go on. In the absence of *p*-AB no synthesis occurs. Under our conditions, a regular increase in the rate of synthesis is seen when the concentration of *p*-AB is increased from 10^{-8} to 10^{-4}M; beyond this concentration the rate remains steady. Results with changes in glutamate concentration have been rather variable. Addition of glutamate is always stimulatory, but there is usually some synthesis without added glutamate.

There has been difficulty in establishing the pH optimum of the system, owing to the high glucolytic activity of the organism. Synthesis is greater, however, at relatively low pH values (pH 4–6).

The action of sulphanilamide and sulphathiazole is clear-cut. Both inhibit the synthesis strongly; the inhibition is completely reversed, in a competitive manner, by increasing the concentration of *p*-AB. The competition has been established over a 10,000-fold range of both sulphonamides.

The results with the sulphonamides on 'folic acid' synthesis provide a complete parallel to the effect of sulphonamides on the growth of this organism.

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An investigation of the properties of *H. pertussis* cultures grown on Bordet-Gengou medium and in liquid semisynthetic medium.

By J. Ungar and P. W. Muggleton

The investigation was undertaken to correlate the yield, viability, virulence in mice, agglutinability and toxin production of four strains of *H. pertussis* in Phase 1. Bordet-Gengou medium or a liquid semisynthetic medium of modified

Hornibrook's formula was used and the properties of the organisms were studied at different stages of growth.

Three separate experiments were performed with the results in good agreement. Although the four strains differed in some of their characteristics, we observed that the optimum time for cultivation on Bordet-Gengou medium was about 48 hr., the strains remaining viable, virulent and agglutinable. On semisynthetic medium the organisms were viable and virulent up to 7 days, but the total yield increased up to 14 days, the toxin in the metabolism fluid also increasing up to 14 days. Freshly isolated strains, such as those used, gave a rather scanty growth during the first 4 days.

A preliminary investigation of the antigenic properties of the various organisms showed that the vaccines prepared from cultures grown on Bordet-Gengou medium gave a marked antibacterial and antitoxic immunity in experimental animals. Vaccines prepared from cultures on the semisynthetic medium produced a similar antibacterial immunity, but the antitoxic immunity was lower. This partial deficiency might be caused by the loss of the soluble toxin in the supernatant fluid during growth of the culture.

A study of the antibiotic phenomena of members of the Actinomycetales.

By M. Lumb

The order Actinomycetales comprises a large group of micro-organisms which show morphological and other characteristics intermediate between bacteria and fungi. Waksman & Henrici (1943) divide the order into three families, Mycobacteriaceae, Actinomycetaceae and Streptomycetaceae.

The Streptomycetaceae are of particular importance to the antibiotic worker as the family includes about thirty-six species which are known to produce antibiotic substances. From seven of these species, twelve antibiotics have been isolated as solid compounds some of which are crystalline.

Fewer antibiotic-producing organisms are found in the Actinomycetaceae, but nevertheless three active species have been reported.

The morphology of many of these organisms has been studied under both surface and submerged culture conditions. There appear to be relationships between morphology and antibiotic production. In the case of *Streptomyces griseus* this work has been followed by biochemical studies of the culture brews throughout the fermentation cycle. It has been found that in submerged culture there is very rapid mycelial development which occurs in the first 20–24 hr. It may be as great as 0·6 % w/v of liquor. During this phase there is (*a*) rapid metabolism of carbohydrate, (*b*) steady utilization of amino-nitrogen, (*c*) fall in O/R potential to a constant value, (*d*) early slight drop in pH followed by a steady rise, and (*e*) little streptomycin formation.

A second phase follows and lasts until the 40–48th hr. of fermentation. It is accompanied by autolysis of mycelium, rise in ammonia N_2 and rapid rise in the biological activity of the culture liquor.

The third and last phase results in continued autolysis with little streptomycin production. There is a drop in streptomycin production measured by maltol estimations, which suggests the formation of some antibiotic other than authentic streptomycin.

Detailed studies have shown that *S. griseus* is a very unstable organism. Its variants differ in their ability to yield streptomycin.

A substance inhibitory to *Saccharomyces logos* and not extracted by the normal Carter (1945) process has been detected in streptomycin liquors. It is possible that it is identical with an active substance identified by Leach, Ford & Whiffen (1947).

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Some properties of Turnip Yellow Mosaic virus. By Roy Markham

Turnip yellow mosaic virus has been isolated from several cruciferous plants. The preparations, which are highly active, contain protein and nucleic acid of the pentose type combined with protein.

While the preparations so obtained are homogeneous on electrophoresis and have a fairly constant composition on ultracentrifugation, they are found to contain two components differing in sedimentation velocity. The two have been isolated nearly pure and their properties examined.

The more slowly sedimenting material has been found to consist of the virus protein alone, while the other component is a nucleo-protein with a high nucleic acid content.

The two components apparently differ only in weight and not in size and both crystallize in the same crystalline form. There is considerable evidence that the nucleic acid of the virus is held inside a spherical shell of protein.

The protein appears to need to be combined with nucleic acid in order to cause infection.

The role of nucleic acid in the antigenic behaviour of Turnip Yellow Mosaic virus. By R. E. F. Matthews

Turnip yellow mosaic virus can be obtained with and without nucleic acid bound in the protein.

It is thus possible to study the role of the nucleic acid in the antigenic behaviour of the virus.

It has been found that the nucleic acid containing virus is highly antigenic. The nucleic acid-free protein is very much less antigenic. Owing to the presence of small amounts of nucleic acid containing virus in the preparations, it has not been possible to determine whether this loss of antigenicity is complete.

In quantitative studies on the reaction of the virus with antibody *in vitro* it has been found that the velocity of the reaction and the amount of antibody which combines with a given weight of virus protein appear to be identical for the nucleoprotein and the nucleic acid-free virus.

The significance of haemoglobin in biological nitrogen fixation.

By John D. Smith

A study of the distribution, localization and concentration of haemoglobin in various types of leguminous root nodules has added strength to the hypothesis that the pigment is intimately concerned in the process of nitrogen fixation. It has been shown that the haemoglobin is localized within the large bacteria-containing cells found in the centre of the nodule. During nodular development haemoglobin does not appear in detectable amounts until this type of cell has been formed. In actively fixing nodules produced by effective strains of *Rhizobium* on various plants the concentration of haemoglobin in these cells varies between 10^{-4} and 5×10^{-4} M (as haematin). Apart from very old or very young nodules the concentration does not vary with age. Haemoglobin could never be detected in Soya nodules produced by the ineffective *Rhizobium* strain 507, however young. The total haematin content of such nodules was much lower than that of effective Soya nodules formed by strain 505, although when grown in pure culture both strains produced approximately equal amounts of haematin.

In an extensive series of experiments it was shown conclusively that the nodule haemoglobin does not function as an oxidation-reduction catalyst in the manner suggested by Virtanen. Methaemoglobin has never been found in nodules, even from darkened plants.

Carbon monoxide, in a concentration such that over 50 % of the haemoglobin was converted into HbCO, did not inhibit the oxygen uptake of detached nodules. Haemoglobin cannot function as a simple oxygen carrier in the respiration of such nodules.

The production of β-alanine from aspartic acid by *Escherichia coli*.

By J. A. Roper and H. McIlwain

Several aspects of the metabolism of pantothenate have been investigated (cf. McIlwain, 1947), including its synthesis by intact cells of *Escherichia coli*. This synthesis appeared to proceed through β-alanine, and we have now investigated the origin of the β-alanine. Decarboxylation of aspartic acid appeared a likely source (cf. Virtanen & Laine, 1937), but the quantities of material involved (pantothenate synthesis proceeded at rates of about 1–3 mμ mol./mg. dry wt. of cells/hr.) precluded direct observation of the decarboxylation by evolution of CO_2 as has been done in other cases by Gale (1945).

We have therefore developed a suitable assay method of β-alanine, using a strain of *Corynebacterium diptheriae* (cf. Schenk, 1943). In this way intact cells of *Escherichia coli* have been shown to produce β-alanine from 0·01 M aspartic acid at rates of 1·0 to 1·5 mμ mol./mg. dry wt./hr. at pH 5. The optimum pH of the enzyme was about 5, and an extract has been obtained which in buffer solutions possessed 60 % of the activity of the whole cells.

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The relationship between the haemagglutinin and infectivity of Influenza and Newcastle Disease viruses. By C. M. CHU

The effects of heating at different temperatures and of different concentrations of formalin on the haemagglutinin and infectivity of influenza and Newcastle disease virus (N.D.V.) are studied. Heating of N.D.V. up to 56° C. for 15 min. only moderately reduced its haemagglutinin and infectivity titres, whereas heating at 58° C. for 15 min. abolished its haemagglutinating activity and sharply reduced its infectivity *in ovo* over a millionfold. Formalin in 0·2 or 0·5 % concentrations completely inactivated the infectivity of N.D.V. in 18 hr., but had no appreciable action on its haemagglutinin over the same period, although a marked decline of haemagglutinin titre occurred in 3 days. Heated or formolized N.D.V., if causing haemagglutination at all, is adsorbed on to and eluted from fowl red cells as does the untreated virus. Influenza B virus (Lee strain) heated to 52 or 54° C. for 15 min. retained its full haemagglutinin titre, but was no longer demonstrably eluted from fowl red cells. At the same time, its haemagglutinin became increasingly inhibited by the 'Francis inhibitor' present in purified human blood group 'O' substance; its enzymic action on the 'Francis inhibitor' was increasingly impaired; and there was a steady drop in infectivity *in ovo*. Formalin in concentrations up to 0·2 % rapidly inactivated the infectivity of influenza B virus, but neither its haemagglutinin titre, nor its eluting power from fowl red cells, nor its behaviour toward the 'Francis inhibitor' was altered within the experimental period of 8 days. Higher formalin concentration, 0·5 %, caused a slow deterioration of influenza haemagglutinin.

It is concluded that heat inactivation of N.D.V. is accompanied by loss of haemagglutinating power; and that of influenza virus, by loss of eluting power, thus destroying its enzymic function. These findings strongly indicate that the complete functioning of the haemagglutinin component of these viruses is essential for their infectivity. Formalin appears to hit at some other components of these viruses which are equally essential for infectivity.

The serological differentiation of staphylococcal bacteriophages.

By Phyllis M. Rountree

A series of thirty-one staphylococcal bacteriophages has been examined by serological means, using sera prepared in rabbits for phage neutralization tests. The results showed that six serological types could be differentiated, the neutralization tests giving clear-cut differences between these types. These serological types could also be correlated with the origins of the phages, with the strains of staphylococci which they attacked, and with certain other characteristics of the phages such as stability and plaque size.

The serological typing of *Staphylococcus pyogenes* and its relationship to bacteriophage reactions.

By Betty C. Hobbs

Further work has been carried out on the serology of staphylococci. The known and suggested types and subtypes hitherto described by Cowan, Christie and Keogh, along with recently isolated strains, have been formed into a series of types by means of absorbed sera.

A comparison is made between the results obtained by both serological and bacteriophage methods of typing for a series of recently isolated strains from widely scattered outbreaks of staphylococcal infection including pemphigus neonatorum, human mastitis, boils and staphylococcal food-poisoning.

Observations are made on the correlation and irregularities shown to exist between the two methods of typing.

Riboflavin enhancement of radioactive phosphate exchange by yeasts.

By Walter J. Nickerson

Through the use of isotopic P^{32} it was found that addition of riboflavin to yeasts metabolizing glucose enhanced their phosphate exchange. This was observed with *Saccharomyces cerevisiae* (bakers' yeast) and with *Candida albicans*. Increases observed ranged from 20 to 200 % over the phosphate exchanged by controls in glucose alone. The effect was also observed with suspensions which had metabolized glucose at 25° C. and were subsequently placed at 4° C. in the presence of P^{32}. The possibility of a surface-acting P-complexing role for riboflavin in phosphate exchange may be suggested.

Since agents (such as dinitrophenol and sodium azide) which are known to inhibit glucose assimilation by yeasts also inhibit phosphate exchange, it was of interest to find that riboflavin promotes a greater assimilation of added glucose by these yeasts as revealed manometrically and by increase in dry weight of the cells.

While it is well known that the presence of external phosphate is not essential for the oxidation and assimilation of glucose by yeast suspensions, it is also known that phosphate exchange and polymerization (as measured by the increase in basophily) are dependent on the presence of external metabolizable substrate. Both exchange of P and polymerization of P by yeasts were found to be enhanced by riboflavin and to be inhibited by dinitrophenol.

It appears then with yeasts, in the presence of phosphate, that agents in concentrations inhibiting glucose assimilation (but not respiration) also inhibit P-exchange and polymerization, while agents enhancing assimilation enhance P-exchange and polymerization.

Some observations on an organism present in sewage (*Bacillus thiocyan oxidans*) which utilizes thiocyanates. By F. C. HAPPOLD and H. J. ROGERS

Happold & Key (1937) described a Gram-negative motile organism isolated from enriched sewage effluent which was able to utilize ammonium thiocyanate as energy source. The organism is autotrophic in the sense that it is able to grow on media containing only ammonium thiocyanate and phosphate. It is, however, able to use organic substances and grows profusely on nutrient agar, producing a greenish diffusible pigment. In the present work washed suspensions of this organism have been examined and when grown on agar containing thiocyanate and phosphate only, the preparations are able to use up to 0·2 % of ammonium thiocyanate. Cultures grown on nutrient medium, whether it contains thiocyanate or not, fail to give active preparations. The time curve for the utilization of thiocyanate by active preparations shows an increasing rate as the concentration of the salt decreases. The optimum temperature for growth of the organism is 22° C. and if cultures are exposed for times as short as 4 hr. to 37° C., then inactive preparations are obtained. These observations might be best interpreted in terms of the selection of active mutants rather than by adaptation of the cells. Preliminary studies of the mechanism of thiocyanate utilization suggest that cyanide-sensitive enzymes are involved. It has so far not proved possible to obtain an active cell-free preparation.

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The examination of bacterial filtrates by paper chromatography. By A. WOIWOD and H. PROOM

This is a preliminary report of the application of partition paper chromatography (Consden, Gordon & Martin, 1944) to a taxonomic survey of the amino-acid metabolism of bacteria. Using sheets of Whatman No. 4 filter-paper, 22 × 18 in., and single dimensional chromatography, we have examined 2- and 10-day filtrates from 50 strains of bacteria grown on a defined medium. *n*-Butanol + acetic acid mixture (Partridge, 1948) has been used as a solvent as it separates the amino-acids into well-defined groups. The medium was an acid-hydrolysate of casein (3 g. TN/l.) with 0·1 % glucose; pH 7·6. This medium gave clear chromatograms uncomplicated by the initial presence of polypeptides. This combination of method and medium gave reproducible results.

When any of the organisms tested grew in this medium the serine spot was decreased in intensity; this was the first effect noted on the chromatogram. All members of the Proteus group produced two polypeptide spots which have R_f values greater than leucine. This did not occur with any of the other groups of organisms examined. *Vibrio comma* characteristically removed completely the basic amino-acids, usually during the first two days of growth. The purpose of this communication is not so much to describe the effects observed as to indicate the usefulness of the method.

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DEMONSTRATIONS

W. Goldie, M. Gordon and K. I. Johnstone. Mutation in a single-cell culture of *Corynebacterium diphtheriae.*

V. D. Allison. Bacteriophage typing of *Staphylococcus pyogenes.*

M. R. Pollock. Bacterial symbiosis based on the combined growth-stimulating and growth-inhibitory properties of long-chain unsaturated fatty acids.

F. W Jelks. An inexpensive substitute for the Petri dish.